V. 58 #2

The Journal of LABORATORY and CLINICAL MEDICINE

The Official Publication of the Central Society for Clinical Research

Volume 58 | Number 2

August, 1961

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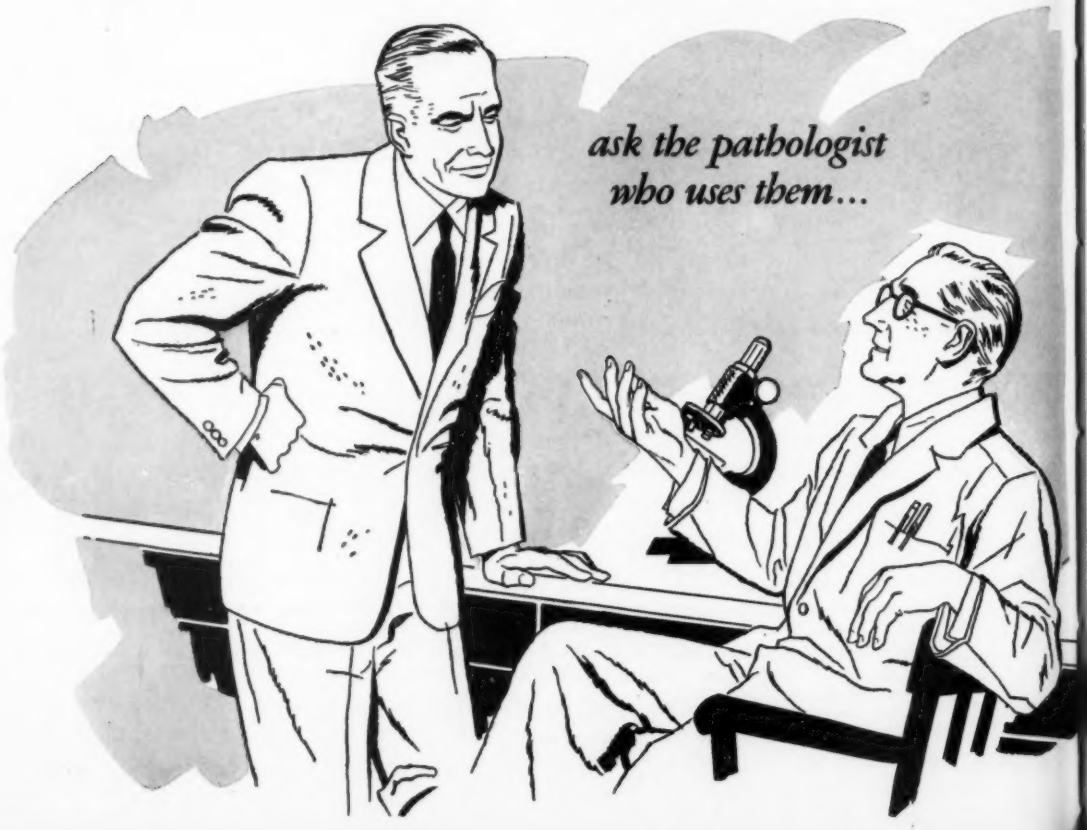

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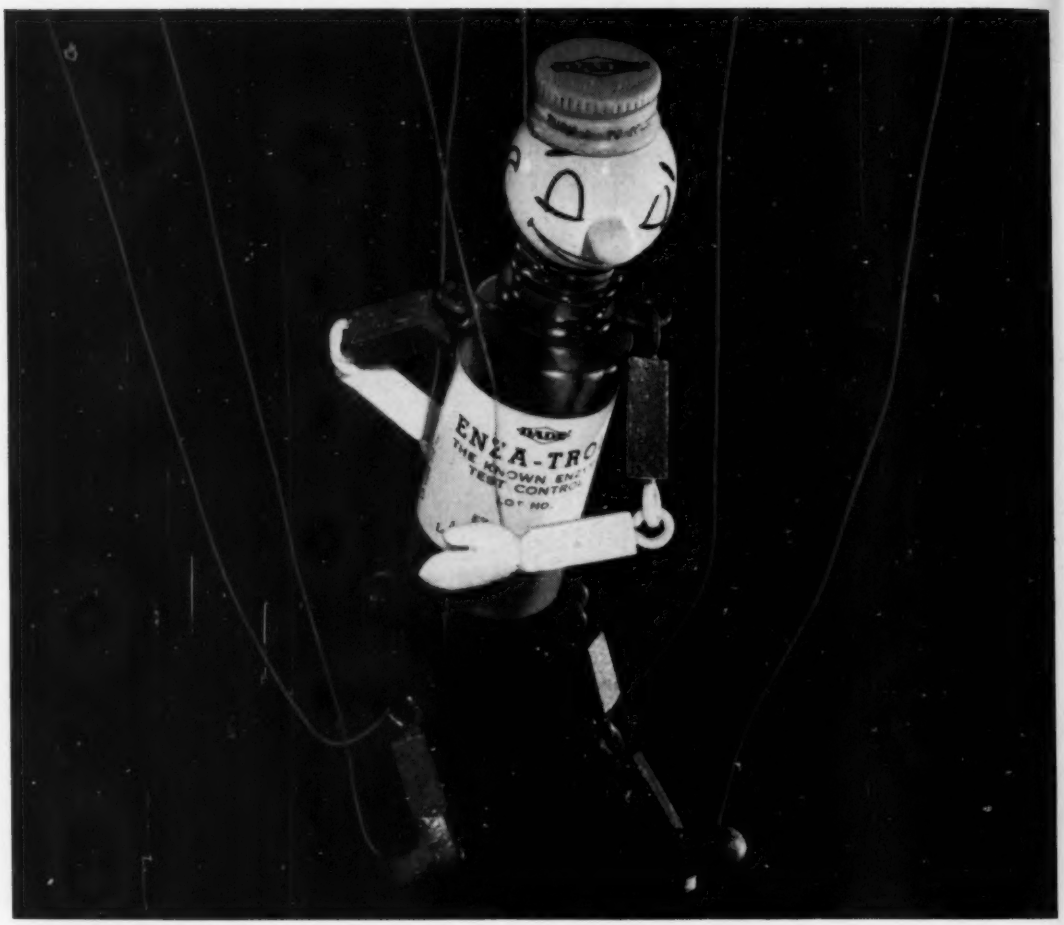

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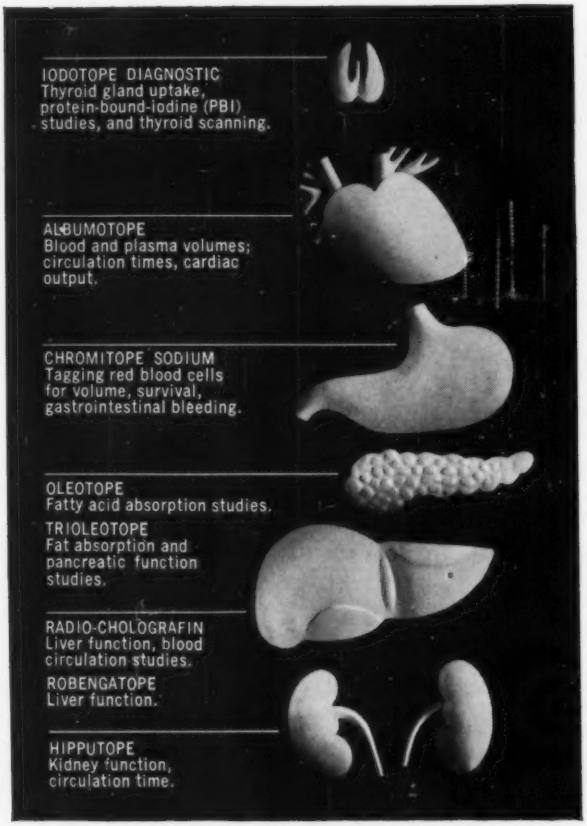

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2. Heiskell, C. L., Fisk, R. T., Florsheim, W. H., Yachi, A., Goodman, J. R., and Carpenter, C. M.: A Simple Method for Quantitation of Serum Beta-Lipoproteins by Means of the Immunocrit, Amer. J. Clin. Path. *35*:222 (March) 1961.

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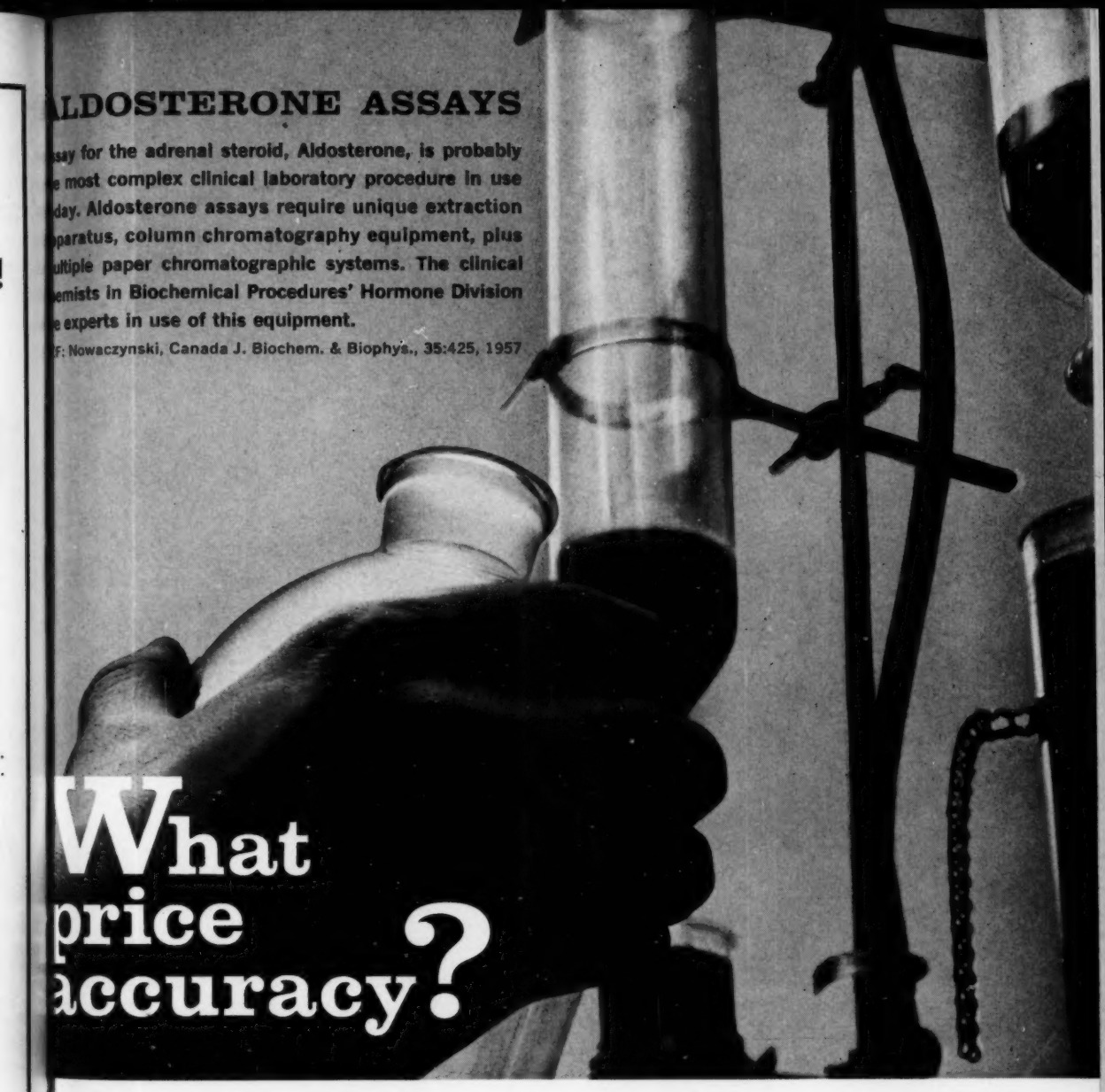

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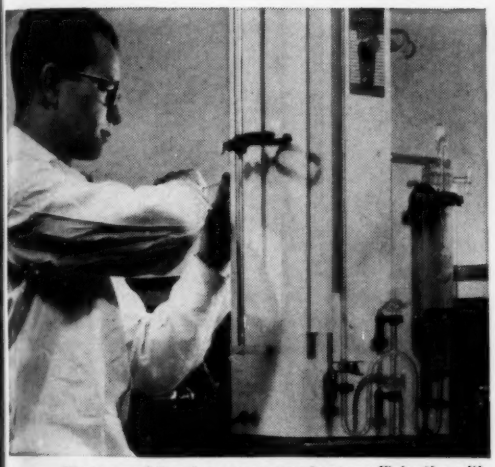

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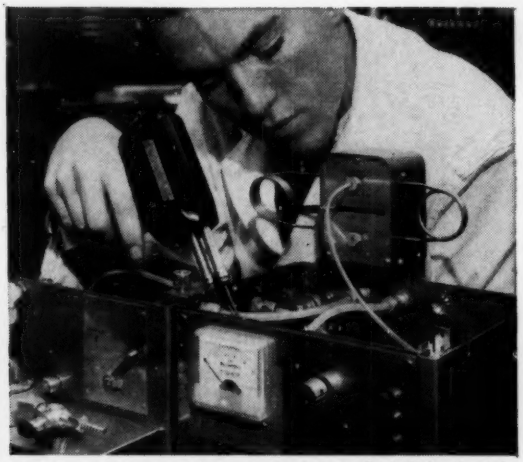

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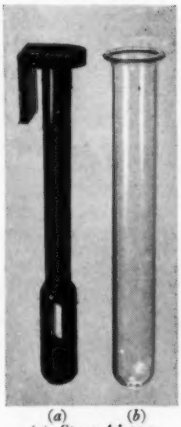
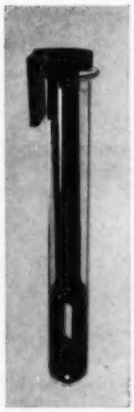


(a) (b)
(a) *Space Adapter*
(b) *Cuvette*



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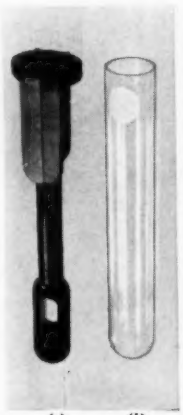
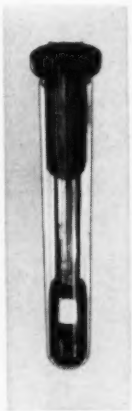


(a) (b)
(a) *Space Adapter*
(b) *Cuvette*



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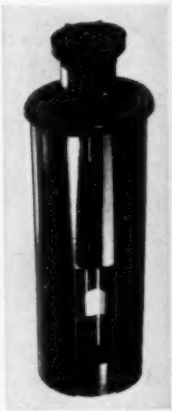

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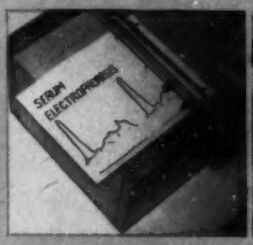

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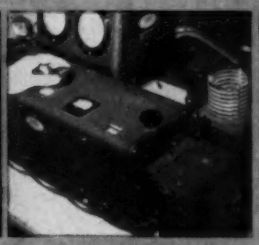

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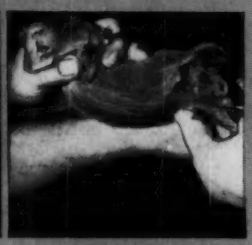

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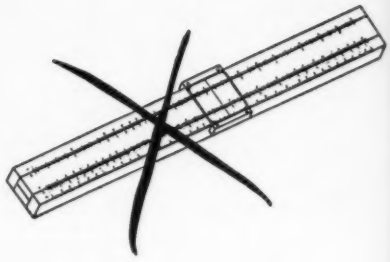

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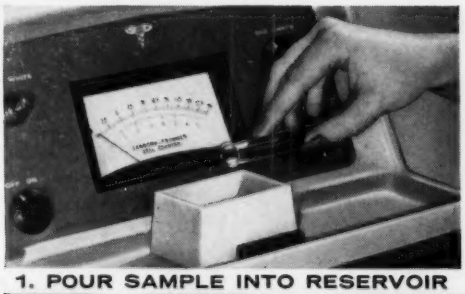

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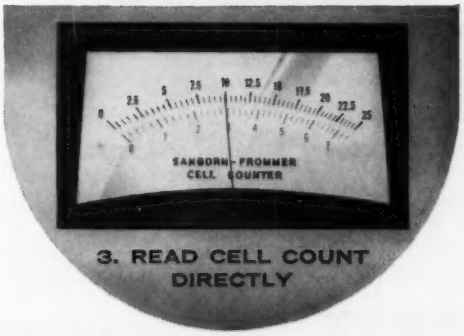
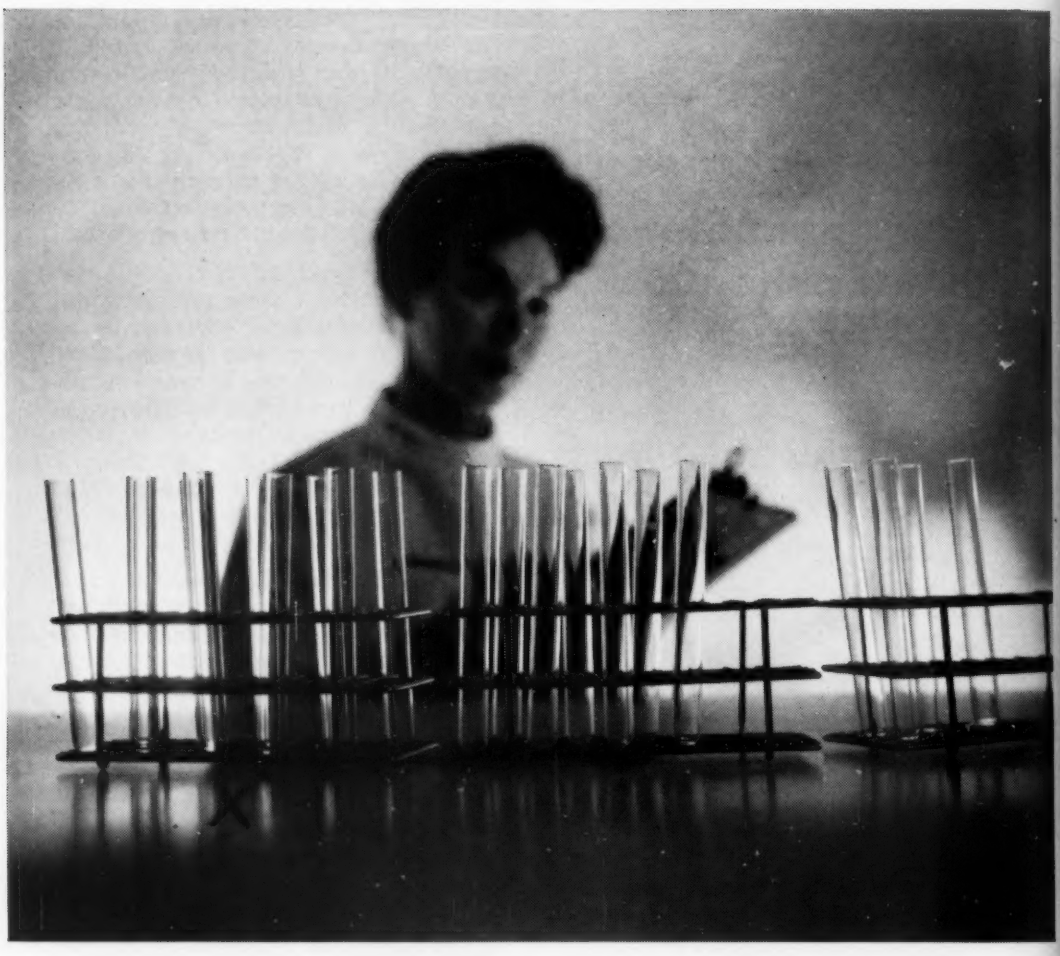


3. READ CELL COUNT DIRECTLY



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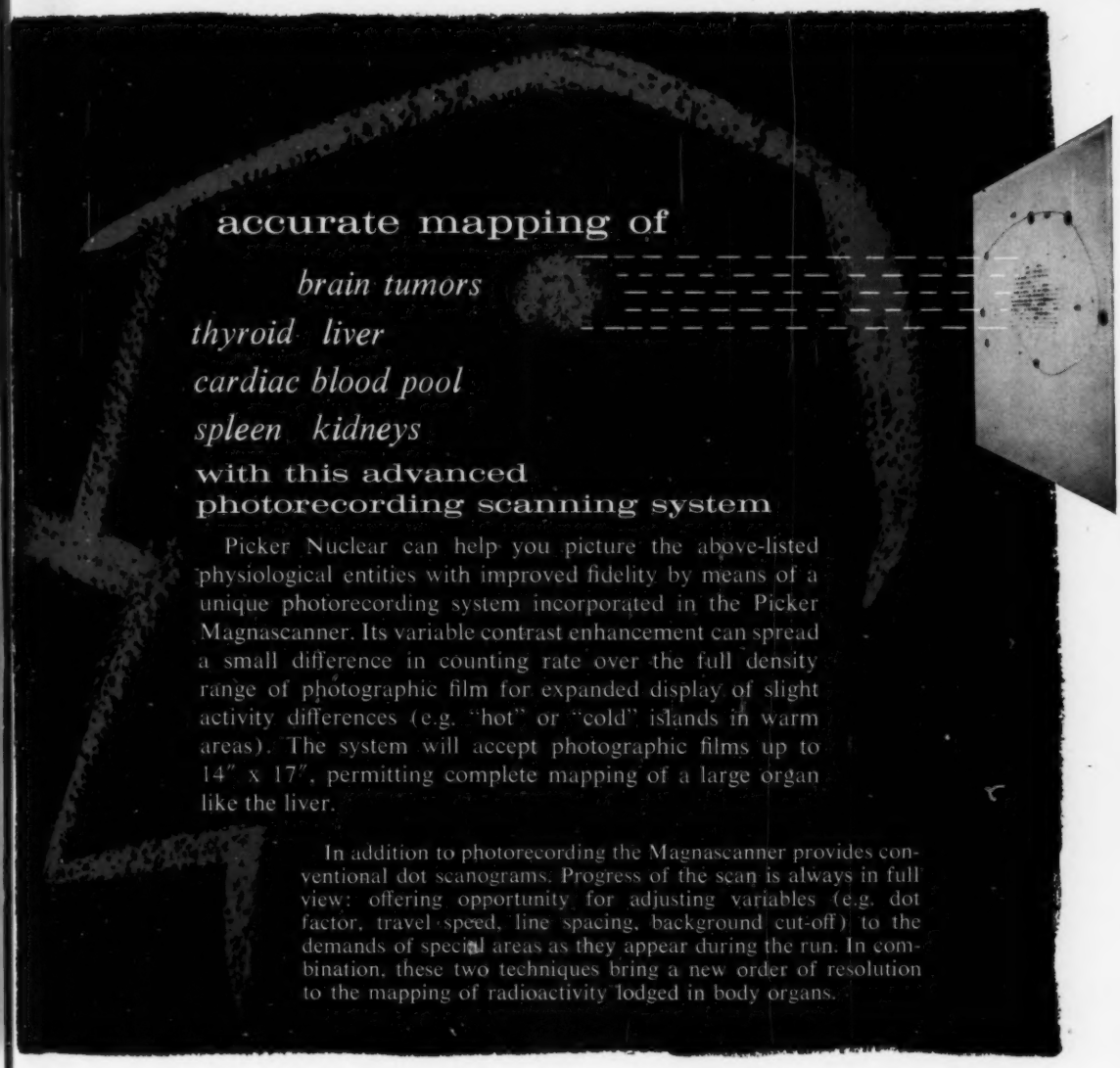

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The Journal of
LABORATORY and CLINICAL MEDICINE

Volume 58 Number 2 AUGUST 1961

Clinical and experimental

An evaluation of maximal water diuresis in chronic renal disease

I. Normal solute intake

CHARLES R. KLEEMAN, M.D.,* DONALD A. ADAMS, M.D.**
and MORTON H. MAXWELL, M.D.*** *Los Angeles, Calif.*
WITH THE TECHNICAL ASSISTANCE OF GARY LUNDE and ROSE TWISS

A systematic investigation of the maximal urine diluting capacity has been undertaken in patients with various types of chronic renal disease. Patients were selected on the basis of a stable state of azotemia. Those with progressive azotemia, active urinary tract infection or obstruction, and congestive heart failure were excluded. Precautions were taken to avoid salt depletion or excess. Diluting capacity was studied under the influence of a maximal sustained water diuresis by the measurements of minimal urinary osmolality, maximal urinary flow, and free water clearance. The observed values were "corrected" to normal renal mass by assuming that inulin clearance was a reasonable estimate of residual functioning renal mass. The corrected values were compared with those of a maximal water diuresis in normal subjects excreting increasing solute loads.

The findings suggest that there is no specific "defect" in urinary dilution in chronic renal disease and that the values are consistent with the increased solute load per residual nephron. In contrast, the maximal urinary concentration (concentrating capacity) was consistently less than would be predicted from the relative osmotic diuresis per nephron.

From the Wadsworth Hospital, Veterans Administration Center, the Department of Medicine, University of California at Los Angeles Medical Center, and the Department of Medicine, Mt. Sinai Hospital, Los Angeles.

Supported by The National Heart Institute, United States Public Health Service Research Grant No. A-1634.

Received for publication July 5, 1960.

*Associate Professor of Medicine, University of California Medical Center; Former Chief, Metabolic Section, Veterans Administration Center. Present position: Chief of Medicine, Mt. Sinai Hospital, Los Angeles.

**Former Senior Resident and Fellow in Metabolism, Veterans Administration Center, and Clinical Instructor in Medicine, University of California Medical Center, Los Angeles.

***Attending Specialist in Medicine, Veterans Administration Center, and Associate Clinical Professor of Medicine, University of California Medical Center, Los Angeles.

It frequently has been stated that the ability to dilute the urine in chronic renal disease is well maintained long after impairment of the concentrating ability has taken place.[1-4] This statement rests primarily on observations of urinary specific gravity following a standard concentration test and/or an oral water load. Although the concentrating defect in chronic renal disease has been carefully and systematically studied,[5-7] only limited information seems available referable to the apparent defect in urinary dilution in humans.[8, 9]

Table I. Data for chronic renal disease patients at height of maximal sustained water diu

Patient	*Surface area (M^2)*	*Diagnosis*	*Serum creatinine (mg. %)*	*C_{in}* (ml./minute)*	*C_{pah}† (ml./minute)*
1. R. G.	1.91	Chronic glomerulonephritis	2.7	38.9	179.0
2. G. A.	1.96	Chronic phyelonephritis	11.4	11.2	38.4
G. A.	1.98		19.5	3.4	7.8
3. F. R.	1.90	Polycystic kidneys	6.5	11.5	31.5
F. R.	1.77		11.0	7.3	21.5
4. J. C.	1.82	Chronic glomerulonephritis	5.4	19.3	67.5
5. C. W.	1.77	Nephrosclerosis	6.5	13.2	40.5
C. W.	1.78		8.3	8.5	22.8
6. A. E.	1.64	Chronic phyelonephritis	18.0	6.9	15.8
7. G. D.	1.69	Chronic phyelonephritis	7.7	8.6	23.5
G. D.	1.63	Nephrosclerosis	15.5	5.4	15.9
8. W. S.	1.56	Nephrosclerosis	5.9	13.3	50.0
9. I. S.	1.77	Chronic phyelonephritis	6.0	11.7	39.0
I. S.	1.80		14.5	5.3	5.7
10. E. S.	1.78	Chronic phyelonephritis	11.0	8.3	16.5
11. L. C.	1.82	Chronic phyelonephritis	4.0	23.8	91.2
L. C.	1.81		3.5	11.6	53.8
12. M. F.	1.64	Chronic glomerulonephritis	4.1	22.1	92.4
13. R. K.	1.59	Chronic glomerulonephritis	7.3	14.7	117.5
14. B. S.	1.81	Chronic phyelonephritis	2.4	17.2	70.7
15. B. T. ♀	1.55	Chronic phyelonephritis	3.5	19.0	150.6
16. H. C.	2.05	Chronic phyelonephritis	11.5	10.4	30.3
17. A. S.	1.69	Chronic phyelonephritis	5.9	12.8	20.4
18. O. S.	1.68	Nephrosclerosis	6.4	13.1	53.8
After 0.5 Gm. Aminophyllin				14.6	45.1
19. S. F.	1.73	Chronic phyelonephritis	3.5	23.1	98.0
20. A. L.	2.05	Polycystic kidneys	7.6	11.0	57.3
21. G. C.	1.62	Chronic phyelonephritis	7.2	12.8	42.8
22. W. B.	1.84	Malignant nephrosclerosis	5.8	11.9	48.1
23. W. H.	1.82	Chronic phyelonephritis	18.1	4.5	8.8
24. P. W.	1.59	Chronic phyelonephritis	4.8	8.5	22.8
25. L. E.	1.83	Chronic glomerulonephritis	3.2	25.5	120.7
26. G. T.	2.03	Malignant nephrosclerosis	7.4	11.8	63.3
27. F. B.	2.02	Chronic glomerulonephritis	4.5	14.6	117.0

*Inulin clearance.
†Para-aminohippurate clearance.

The present investigation is an evaluation of the parameters of maximal water diuresis in nonedematous patients with moderate to severe chronic renal insufficiency.

Materials and methods

Thirty-three experiments were performed on 27 azotemic patients with stable chronic renal disease. No attempt was made to select a specific renal disorder for study, the major requirements being the absence of congestive heart failure, edema, active urinary tract infection, or clinically recognizable depletion of extracellular volume or electrolytes. The

water diuresis (all data corrected to 1.73 M^2)

ah† inute)	*Urinary flow* (ml./minute)	*Urinary osmolality* (mOsm./Kg.)	*Serum osmolality* (mOsm./Kg.)	*Solute excretion* (mOsm./minute)	C_{osm}‡ (ml./minute)	C_{H_2O}§ (ml./minute)	*Pitressin concentration urinary osmolality* (mOsm./Kg.)
9.0	6.57	77	295	.506	1.72	4.85	356
8.4	3.59	127	306	.456	1.49	2.10	280
7.8	2.26	209	311	.472	1.52	0.74	287
1.5	3.82	169	303	.646	2.13	1.69	345
1.5	2.30	190	290	.437	1.50	0.80	345
7.5	4.20	131	296	.550	1.86	2.34	—
0.5	4.24	145	288	.615	2.14	2.10	—
2.8	3.79	179	312	.678	2.17	1.62	322
5.8	3.23	200	305	.646	2.12	1.11	309
3.5	2.64	203	306	.536	1.75	0.89	—
5.9	2.96	195	294	.577	1.96	1.00	—
0.0	4.32	153	261	.661	2.53	1.79	(sp. gr.: 1.016)
9.0	3.61	192	300	.693	2.31	1.30	294
5.7	1.41	218	316	.307	0.97	0.44	342
6.5	4.34	194	300	.842	2.81	1.53	—
1.2	9.75	157	293	1.530	5.22	4.53	392
3.8	2.94	115	288	.338	1.17	1.77	371
2.4	6.33	134	284	.848	2.99	3.34	370
7.5	3.03	228	315	.691	2.19	0.84	325
0.7	2.25	149	297	.335	1.13	1.12	369
0.6	2.64	230	294	.607	2.06	.58	—
0.3	2.96	168	311	.497	1.60	1.36	(sp. gr.: 1.008)
0.4	5.70	151	283	.861	3.04	2.66	314
3.8	2.63	147	274	.387	1.41	1.22	—
5.1	3.40	148	262	.503	1.92	1.48	—
8.0	5.10	183	309	.933	3.02	2.08	418
7.3	2.69	251	304	.675	2.22	0.47	(sp. gr.: 1.010)
2.8	2.94	160	287	.471	1.64	1.30	(sp. gr.: 1.009)
8.1	2.41	169	300	.407	1.34	1.07	330
8.8	2.32	272	325	.631	1.94	0.38	281
2.8	3.65	179	292	.653	2.24	1.41	292
0.7	4.62	122	268	.564	2.10	2.52	383
3.3	3.59	176	304	.632	2.08	1.51	369
7.0	3.13	193	312	.604	1.94	1.19	383

‡Osmolar clearance.
§Free water clearance.

Table II. Data for normal subjects at height of sustained water diuresis (all data

	Patient	*Surface area (M^2)*	*C_{in} (ml./minute)*	*C_{pah} (ml./minute)*	*Urinary flow (ml./minute)*	*Urinary osmolality (mOsm./Kg.)*
1.	R. S.	2.05	153	—	17.2	81
2.	J. C.	1.63	143	571	22.6	54
3.	A. M.	1.87	124	610	18.5	53
4.	D. B.	2.20	134	580	20.2	51
5.	M. S.	1.96	93	—	19.4	85
	M. S.	1.96	113	—	20.3	59
6.	J. Y.	1.59	132*	—	23.4	74
7.	H. W.	1.81	126	429	18.9	114
8.	J. K.	1.82	106	484	12.0	67
9.	E. V.	1.50	177	846	21.5	58
	E. V.	1.50	138*	—	15.6	48
10.	W. G.	1.46	110	—	14.3	57
11.	G. W. †	2.10	137*	—	18.2	57
	G. W.	2.10	160	506	18.1	62
	G. W.	2.07	129	612	13.6	49
12.	C. C. †	1.83	178*	—	18.1	51
	C. C.	1.78	128	486	15.5	57
13.	R. M. †	1.92	166*	—	14.2	88
	R. M.	1.92	131*	—	17.1	83
	Mean values (19)	1.85	129	569	17.8	66

*Creatinine clearance.
†Diabetes insipidus patients.

diagnoses are listed in Table I. All patients were ambulatory and eating well. The diets contained 40 to 80 Gm. of protein and 4 to 8 Gm. of sodium chloride. The lower levels of protein intake were ingested by the most azotemic patients. All experiments were conducted with the patients supine and in the postprandial state.

Dilution test. Following the oral ingestion of 1,000 ml. of water, this positive state of hydration was maintained for a 5 hour period by the continuous intravenous infusion of 2.5 per cent dextrose in water equal to the concomitant urine volume. A sustaining infusion of inulin and para-aminohippurate (PAH) was maintained after a priming dose. Urine specimens were obtained by spontaneous voiding; patients who had significant residual volumes in the bladder or who experienced difficulty in voiding were catheterized during the experiment. Four to 6 clearance periods of 20 to 30 minutes duration were utilized at the height of sustained water diuresis between the third and fifth hours, with blood samples drawn at the mid-point of each period. Serum and urine were analyzed for inulin, PAH, creatinine, sodium, and freezing-point depression by methods previously reported.[10, 11]

Concentration test. The patients ingested no water after 9:00 P.M. At 7:00 A.M. the next day they emptied their bladders, and a 4 hour infusion of aqueous Pitressin at the rate of 260 mu. per hour was started. The patients voided hourly for 4 hours and the volume, specific gravity, and osmolality of the last specimen were measured. Breakfast was withheld during the test.

Normal controls. The subjects with normal renal function who were utilized for comparison were the following:

corrected to 1.73 M^2)

Serum osmolality (mOsm./Kg.)	*Solute Excretion (mOsm./ minute)*	C_{osm} *(ml./minute)*	C_{H_2O} *(ml./minute)*	C_{H_2O} *per 100 ml. GFR*	*Urinary flow per 100 ml. GFR*	*Predicted solute excretion per normal* C_{in}
290	1.39	4.8	12.4	8.1	11.2	1.17
270	1.22	4.5	18.1	13.9	15.8	1.22
281	.98	3.5	15.0	12.1	14.9	.98
273	1.03	3.8	16.4	12.2	15.1	1.03
278	1.65	5.8	13.6	14.6	20.8	2.20
284	1.20	4.2	16.1	14.3	19.0	1.31
287	1.73	6.0	17.4	13.2	17.7	1.73
285	2.16	7.6	11.3	9.0	15.0	2.16
274	.80	2.9	9.1	8.6	11.3	.94
269	1.25	4.6	16.9	9.5	12.2	1.25
274	.75	2.7	12.9	9.3	11.3	.75
279	.82	2.9	11.4	10.4	13.0	.82
287	1.04	3.6	14.6	10.6	13.3	1.04
287	1.12	3.9	14.2	8.9	11.3	1.12
264	.67	2.5	11.1	8.6	10.5	.67
299	.92	3.1	15.0	8.4	10.2	.92
266	.88	3.3	12.2	9.5	12.1	.88
281	1.25	4.4	9.8	5.9	8.5	1.25
300	1.42	4.7	12.4	9.5	13.1	1.42
280	1.17	4.1	13.7	10.3	13.4	1.21

1. THOSE WHO DURING OTHER STUDIES WERE UNDERGOING A MAXIMAL WATER DIURESIS PERFORMED IN A COMPARABLE MANNER. In some subjects hypotonic mannitol, a mercurial, and/or Aminophyllin were administered during the water diuresis to increase acutely the rate of solute excretion.

2. THE SIGNIFICANT DATA FROM THE 3 PATIENTS WITH DIABETES INSIPIDUS WHO WERE UNDERGOING A MAXIMAL WATER DIURESIS ARE PRESENTED IN TABLES II AND III.

3. NORMAL SUBJECTS OR PATIENTS WITH DIABETES INSIPIDUS ON WHOM ADEQUATE DATA DURING A MAXIMAL SUSTAINED WATER DIURESIS WERE AVAILABLE FROM THE LITERATURE.[10, 12-14]

By combining the data in subjects from Groups 1, 2, and 3 above, a total of 69 control experiments on these 58 subjects was obtained.

In order to compare the parameters of maximal water diuresis (free water clearance, urinary flow, and minimal urinary osmolality) of the patients with chronic renal disease with those of the normal subjects, it is essential that the comparison be made at equivalent rates of solute excretion, or, more correctly, at equivalent rates per unit of functioning nephron mass. If the kidney with a markedly reduced functional mass is excreting a normal solute load, then each remaining nephron is exposed to a distinct osmotic load. The magnitude of this load referable to normal renal mass would be as follows:

$$\frac{\text{"normal" functional renal mass}}{\text{observed functional renal mass}} \times \text{observed rate of solute excretion (mOsm. per minute)} = \text{predicted rate of solute excretion with normal renal mass.}$$

Table III. Water diuresis during increasing solute excretion in normal subjects (al

Patient	*Mechanism by which solute excretion was increased*	*Surface area (M^2)*	*C_{in} (ml./minute)*	*C_{pah} (ml./minute)*	*Urinary flow (ml./minute)*	*Urinary osmolality (mOsm./Kg.)*
1. R. A.	3% mannitol	1.70	143	575	29.7	119
2. D. A.	3% mannitol	2.04	90	424	20.6	155
3. A. K.	3% mannitol	2.01	100	561	17.8	131
A. K.	3% mannitol + 0.5 Gm. Aminophyllin	2.01	106	676	24.9	199
4. J. N.	2 ml. Hg I.V. + 3% mannitol	1.93	123	—	30.7	142
J. N.	After 0.5 Gm. Aminophyllin	1.93	126	—	42.6	155
5. F. P.	2.5% glucose + 0.5 Gm. Aminophyllin	1.92	206	741	30.1	128
6. L. L.	2.5% glucose + 2 ml. Hg I.V. + 0.5 Gm. chlorothiazide, P.O.	1.75	148	752	24.9	103
7. O. S.	2.5% glucose + 0.5 Gm. Aminophyllin	1.86	140	722	28.8	8
	Mean values (9)	1.89	131	636	27.8	13

If the rate of glomerular filtration (GFR) is a reasonable approximation of functional mass, then the left-hand side of the above equation can be written:

$$\frac{\text{normal GFR*}}{\text{observed GFR}} \times \text{observed rate of solute excretion.}$$

This equation has been utilized in the present study to calculate all the derived data (Table IV). Its limitations will be discussed subsequently. All the observed data have been corrected to 1.73 M^2. The urinary flow (V) and free water clearance (C_{H_2O}) are expressed per 100 ml. of glomerular filtrate, i.e.,

$$\frac{V}{C_{in}} \times 100 \text{ and } \frac{C_{H_2O}}{C_{in}} \times 100.$$

Results

Observed data (Tables I, II, and III, and Figs. 1, 2, and 3). The severity of the impairment in renal hemodynamics and the blunted response to the sustained water load is evident from the data in Table I. When compared to the results obtained with normal subjects from this laboratory and with the literature (Tables II and III, Figs. 1, 2, and 3), the magnitude of the alteration in water diuresis can be appreciated. The observed urinary flow and the C_{H_2O} were always much decreased in the chronic renal disease patients, and the observed minimal osmolality variably but consistently increased.

The maximal urinary concentrations attained during the standard intravenous Pitressin test on patients with chronic renal disease are listed in the last column of Table I. When tested, all patients but Nos. 2 and 23 were able

*In the present study, normal glomerular filtration rate per 1.73 M^2 of body surface area has been considered 124 ml. per minute for men and 109 ml. per minute for women.[15] For uniformity, the rate of solute excretion in the normal subjects and of those in the literature have also been corrected for these filtration rates (Tables II and III).

data corrected to 1.73 M^2)

Serum osmolality (mOsm./Kg.)	*Solute excretion (mOsm./minute)*	C_{osm} *(ml./minute)*	C_{H_2O} *(ml./minute)*	C_{H_2O} *100 ml. GFR*	*Urinary flow per 100 ml. GFR*	*Predicted solute excretion per normal* C_{in}
267	3.53	13.2	16.5	11.5	20.8	3.06
273	3.19	11.7	8.9	9.9	22.9	4.39
285	2.33	8.2	9.6	9.6	17.8	2.89
289	4.96	17.1	7.8	7.4	23.5	5.80
287	4.36	15.2	15.5	12.6	25.0	4.36
286	6.60	23.1	19.5	15.4	33.8	6.60
289	3.85	13.3	16.8	8.2	14.6	2.32
274	2.56	9.3	15.6	6.3	16.8	2.14
282	2.45	8.7	20.1	14.4	20.6	2.17
281	3.76	13.3	14.5	10.6	21.8	3.75

to concentrate to an osmolality at least equivalent to that of the plasma, and always significantly above the maximal urinary dilution (mean of 359 as compared to 174 mOsm. per liter, respectively). Therefore, in this group of patients no example of "fixed" osmolality (isosthenuria) was observed. Cases Nos. 2 and 23 may be mild examples of "acquired nephrogenic diabetes insipidus."[16, 17]

When the maximal urinary flows of the patients with chronic renal disease were expressed per 100 ml. of glomerular filtrate, they greatly exceeded the comparable values of the normal subjects (mean of 31.3 as compared with 14.7 ml. per minute, respectively) (Table II). Despite this, the ability of the patients to maximally dilute their urine was definitely impaired (mean minimal osmolality: 174 as compared to 66 mOsm. per liter, respectively). When all the available data on these parameters of maximal water diuresis are plotted against the predicted rate of solute excretion for normal renal mass (GFR) (Figs. 4 and 5), the apparent differences in maximal urinary flow and minimal urinary osmolality between the normal individuals and the patients disappear, i.e., with this correction, the parameters of maximal water diuresis were quantitatively comparable in both groups.

Urinary flow per 100 ml. GFR, as might be predicted, increased linearly as solute load increased (Fig. 4). Free water clearance (C_{H_2O}) per 100 ml. GFR, however, showed no such simple relationship (Fig. 6). When renal hemodynamics and solute excretion are relatively constant, the free water clearance is inversely related to the level of circulating antidiuretic hormone (ADH).[2, 18, 19] We have assumed that the water-loaded recumbent subject in the present

Table IV. Derived data from chronic renal disease subjects at height of maximal sustained water diuresis (all data corrected to 1.73 M^2)

Patient	*Diagnosis*	*C_{H_2O} per 100 ml. GFR*	*Urinary flow per 100 ml. GFR*	*Predicted solute excretion per normal C_{in}*	*% GFR* of normal*	*% RPF† of normal*
1. R. G.	Chronic glomerulonephritis	12.4	16.9	1.61	31.4	27.4
2. G. A.	Chronic pyelonephritis	18.7	32.1	5.05	9.0	5.9
G. A.	Chronic pyelonephritis	21.8	66.5	17.21	2.7	1.2
3. F. R.	Polycystic kidneys	14.7	33.2	6.96	9.3	4.8
F. R.	Polycystic kidneys	11.0	31.5	7.42	5.8	3.3
4. J. C.	Chronic glomerulonephritis	12.1	21.8	3.53	15.5	10.3
5. C. W.	Nephrosclerosis + polycystic kidneys	15.9	32.1	5.78	10.6	6.2
C. W.	Nephrosclerosis + polycystic kidneys	19.1	44.6	9.89	6.9	3.5
6. A. E.	Chronic pyelonephritis	16.1	46.8	11.61	5.6	2.4
7. G. D.	Chronic pyelonephritis + nephrosclerosis	10.3	30.7	7.73	6.9	3.6
G. D.	Chronic pyelonephritis + nephrosclerosis	18.5	54.8	13.25	4.4	2.4
8. W. S.	Nephrosclerosis	13.5	32.5	6.16	10.4	7.6
9. I. S.	Chronic pyelonephritis	11.1	30.9	7.34	9.4	6.0
I. S.	Chronic pyelonephritis	8.3	26.6	7.18	4.3	0.87
10. E. S.	Chronic pyelonephritis	18.4	52.3	12.58	6.6	2.5
11. L. C.	Chronic pyelonephritis	19.0	41.0	7.97	19.2	13.9
L. C.	Chronic pyelonephritis	15.3	25.3	3.61	9.4	8.2
12. M. F.	Chronic glomerulonephritis	15.1	28.6	4.76	17.1	14.1
13. R. K.	Chronic glomerulonephritis	5.7	20.6	5.83	11.9	18.0
14. B. S.	Chronic pyelonephritis	6.5	13.1	2.41	13.8	10.8
15. B. T. (♀)	Chronic pyelonephritis	3.1	13.9	3.48	17.4	25.4
16. H. C.	Chronic pyelonephritis	13.1	28.5	5.93	8.3	4.6
17. A. S.	Chronic pyelonephritis	20.8	44.5	8.34	10.3	3.1
18. O. S.	Nephrosclerosis	9.3	20.1	3.66	10.6	8.2
After 0.5 Gm. Aminophyllin		10.1	23.3	4.27	11.8	6.9
19. S. F.	Chronic pyelonephritis	9.0	22.1	5.01	18.6	15.0
20. A. L.	Polycystic kidneys	4.3	24.5	7.61	8.9	8.8
21. G. C.	Chronic pyelonephritis	10.2	23.0	4.56	10.3	6.5
22. W. B.	Malignant nephrosclerosis	9.0	20.3	4.24	9.6	7.4
23. W. H.	Chronic pyelonephritis	8.4	51.6	17.39	3.6	1.3
24. R. W.	Chronic pyelonephritis	12.9	42.9	7.43	6.9	3.5
25. L. E.	Chronic glomerulonephritis	9.9	18.1	2.74	20.6	18.5
26. G. T.	Malignant nephritis + chronic pyelonephritis	12.8	30.4	6.64	9.5	9.7
27. F. B.	Chronic glomerulonephritis	8.2	21.4	5.13	11.8	17.9

*Normal GFR (C_F): 124, 109.[15]
†Normal RPF: 654, 592.

study is in a state of physiologic diabetes insipidus, but it is possible that, in some, a small but significant quantity of ADH was still present in the circulating blood during the water load. This could account in part for the scatter of points in Fig. 6. Nevertheless, the distribution of points representing the patients with renal disease, with few exceptions, could not be differentiated from those representing the normal subjects.

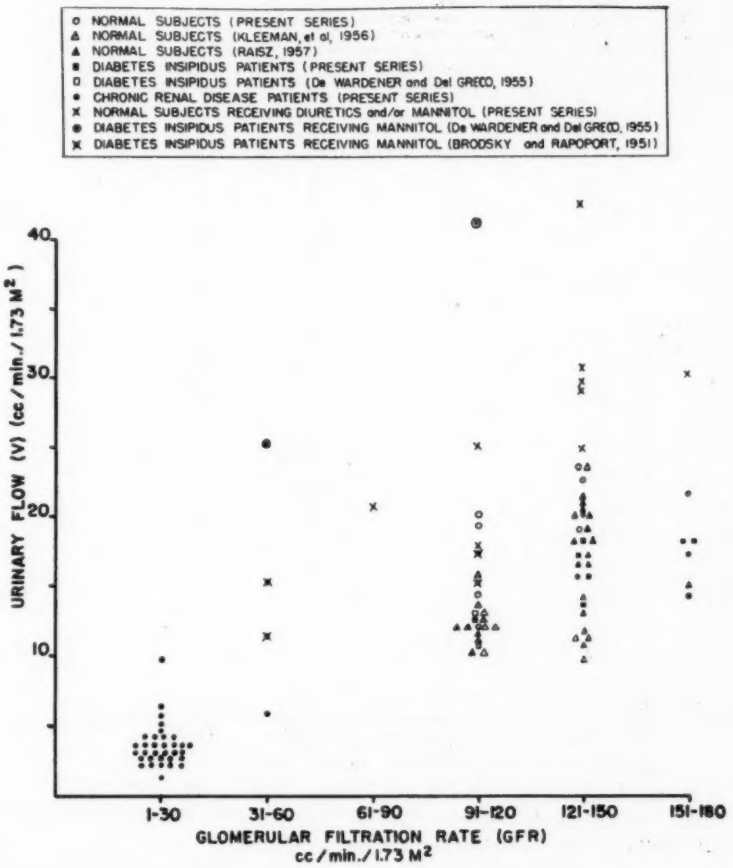

NORMAL SUBJECTS (PRESENT SERIES)
NORMAL SUBJECTS (KLEEMAN, et al, 1956)
NORMAL SUBJECTS (RAISZ, 1957)
DIABETES INSIPIDUS PATIENTS (PRESENT SERIES)
DIABETES INSIPIDUS PATIENTS (De WARDENER and Del GRECO, 1955)
CHRONIC RENAL DISEASE PATIENTS (PRESENT SERIES)
NORMAL SUBJECTS RECEIVING DIURETICS and/or MANNITOL (PRESENT SERIES)
DIABETES INSIPIDUS PATIENTS RECEIVING MANNITOL (De WARDENER and Del GRECO, 1955)
DIABETES INSIPIDUS PATIENTS RECEIVING MANNITOL (BRODSKY and RAPOPORT, 1951)
URINARY FLOW (V) (cc / min. / 1.73 M²)
40
30
20
10
1-30
31-60
61-90
91-120
121-150
151-180
GLOMERULAR FILTRATION RATE (GFR)
cc / min. / 1.73 M²

Fig. 1. Relationship between observed maximal urinary flow and the level of renal function, as measured by the glomerular filtration rate.

In normal subjects, within the physiologic range of solute excretion (<2mOsm. per minute), C_{H_2O} progressively increases with the rate of solute excretion.[2, 10, 14, 18, 20] This is evident from the points below 2 mOsm. per minute in Fig. 6. Between 2 and 10 mOsm. per minute there is no evidence *from these grouped data* of a further increment in C_{H_2O} in either the normal subjects or the patients. Because of the small number of observations, nothing can be said about the C_{H_2O} above 10 mOsm. per minute. Minimal urinary osmolality increased almost asymptotically toward plasma osmolality as predicted solute

excretion increased (Fig. 5). However, it appeared that, regardless of the magnitude of the excreted solute, some dilution of the glomerular filtrate could always be attained.

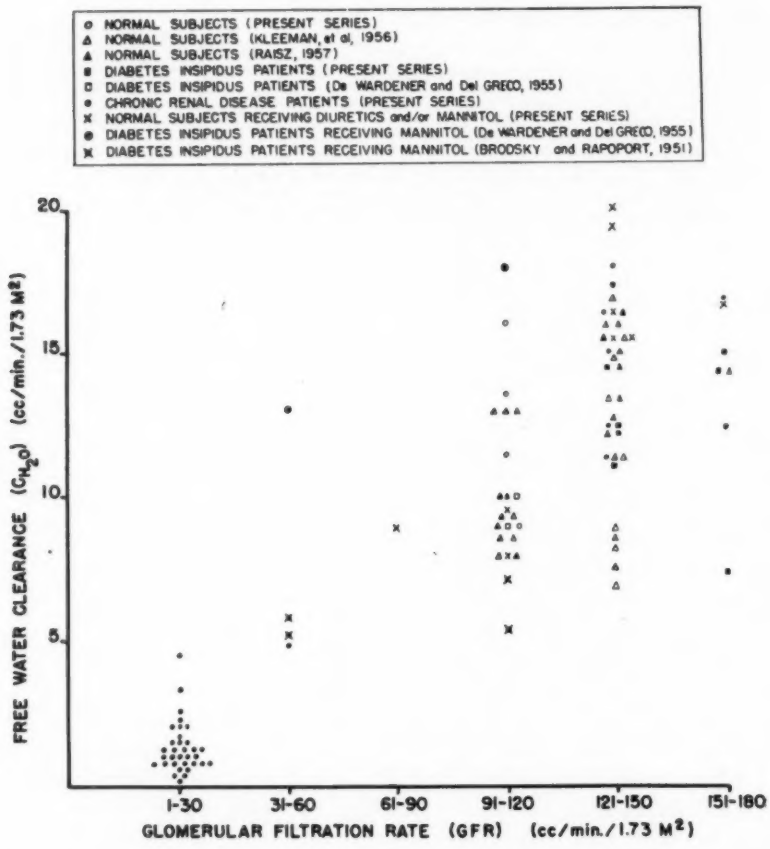

NORMAL SUBJECTS (PRESENT SERIES)
NORMAL SUBJECTS (KLEEMAN, et al, 1956)
NORMAL SUBJECTS (RAISZ, 1957)
DIABETES INSIPIDUS PATIENTS (PRESENT SERIES)
DIABETES INSIPIDUS PATIENTS (De WARDENER and Del GRECO, 1955)
CHRONIC RENAL DISEASE PATIENTS (PRESENT SERIES)
NORMAL SUBJECTS RECEIVING DIURETICS and/or MANNITOL (PRESENT SERIES)
DIABETES INSIPIDUS PATIENTS RECEIVING MANNITOL (De WARDENER and Del GRECO, 1955)
DIABETES INSIPIDUS PATIENTS RECEIVING MANNITOL (BRODSKY and RAPOPORT, 1951)
FREE WATER CLEARANCE (C_{H_2O}) (cc/min./1.73 M²)
20
15
10
5
1-30
31-60
61-90
91-120
121-150
151-180
GLOMERULAR FILTRATION RATE (GFR) (cc/min./1.73 M²)

Fig. 2. Relationship between free water clearance (C_{H_2O}) and the level of renal function, as measured by the glomerular filtration rate.

Discussion

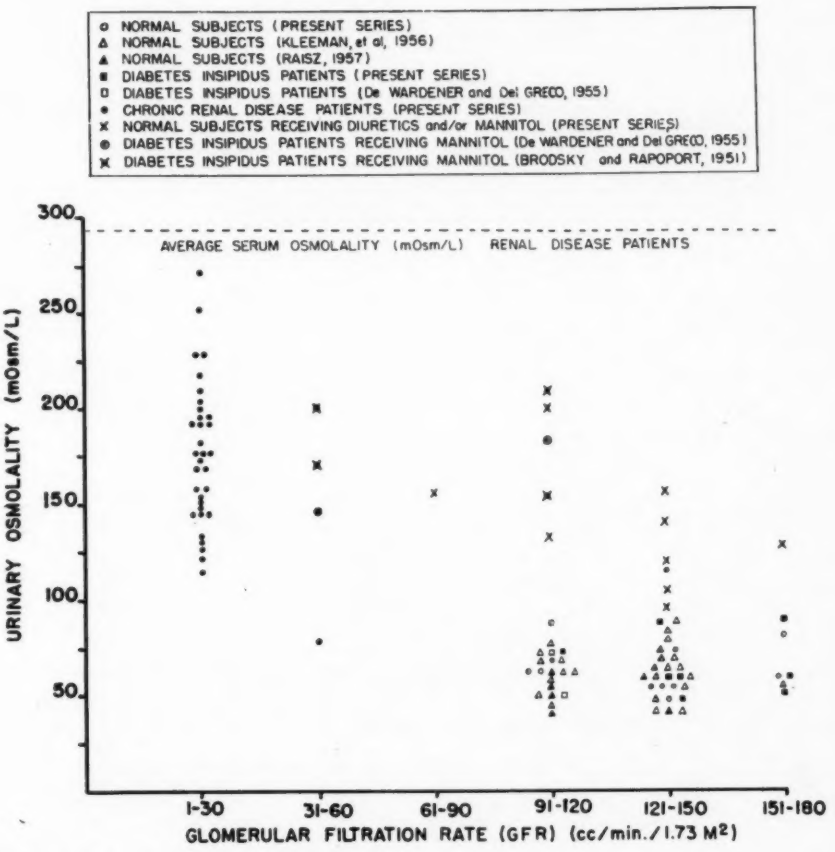
The approach to the problem of water diuresis in chronic renal disease utilized in the present investigation makes the following assumptions:

1. The remaining nephrons are relatively uniform in function and normal with respect to "glomerulo-tubular balance."

2. Inulin clearance measures GFR in chronic renal disease,[21] and the latter is a reasonable approximation of renal functioning mass.

Oliver's microdissection studies on the diseased kidney[21] have clearly shown that the residual nephrons are far from uniform in structure. This would mean that some nephrons would actually be carrying a greater solute

load than assumed, while others would be carrying less. The present derived data, therefore, represent only the over-all function of a heterogenous nephron population.*


o NORMAL SUBJECTS (PRESENT SERIES)
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▲ NORMAL SUBJECTS (RAISZ, 1957)
■ DIABETES INSIPIDUS PATIENTS (PRESENT SERIES)
□ DIABETES INSIPIDUS PATIENTS (De WARDENER and Del GRECO, 1955)
• CHRONIC RENAL DISEASE PATIENTS (PRESENT SERIES)
× NORMAL SUBJECTS RECEIVING DIURETICS and/or MANNITOL (PRESENT SERIES)
⊕ DIABETES INSIPIDUS PATIENTS RECEIVING MANNITOL (De WARDENER and Del GRECO, 1955)
✕ DIABETES INSIPIDUS PATIENTS RECEIVING MANNITOL (BRODSKY and RAPOPORT, 1951)
AVERAGE SERUM OSMOLALITY (mOsm/L) RENAL DISEASE PATIENTS
URINARY OSMOLALITY (mOsm/L)
300
250
200
150
100
50
1-30
31-60
61-90
91-120
121-150
151-180
GLOMERULAR FILTRATION RATE (GFR) (cc/min./1.73 M²)

Fig. 3. Relationship between minimal urinary osmolality and the level of renal function, as measured by the glomerular filtration rate.

At present, there is no way of properly assessing whether the rate of glomerular filtration (inulin clearance) is a reasonable approximation of the remaining functional renal mass. To our knowledge, no other test of renal function is any more reliable for this purpose. Great care was taken to select patients whose state of water and electroylte balance and cardiac function were normal, so that structural, rather than "functional," reductions in inulin clearance could be reasonably assured. Despite this, the inulin clearance may be only a rough approximation of functioning nephrons. The derived data and the conclusions drawn must be accepted with this limitation in mind.

*In chronic renal disease and experimental reductions in renal mass, the glomerular filtration per nephron may actually be greater than normal. If this is a general occurrence in the patients studied, the derived value for the predicted rate of solute excretion is less than the actual magnitude of the osmotic load per nephron; this possibility cannot be excluded.

In normal subjects, for any given level of glomerular filtration, the rate of solute excretion influences the urinary flow and the minimal urinary osmolality (i.e., as solute load increases, urinary flow and osmolality increase).[10, 14, 18, 20] Therefore, in chronic renal disease, the excretion of "corrected" solutes (see above) must be utilized in interpreting the parameters of water diuresis.

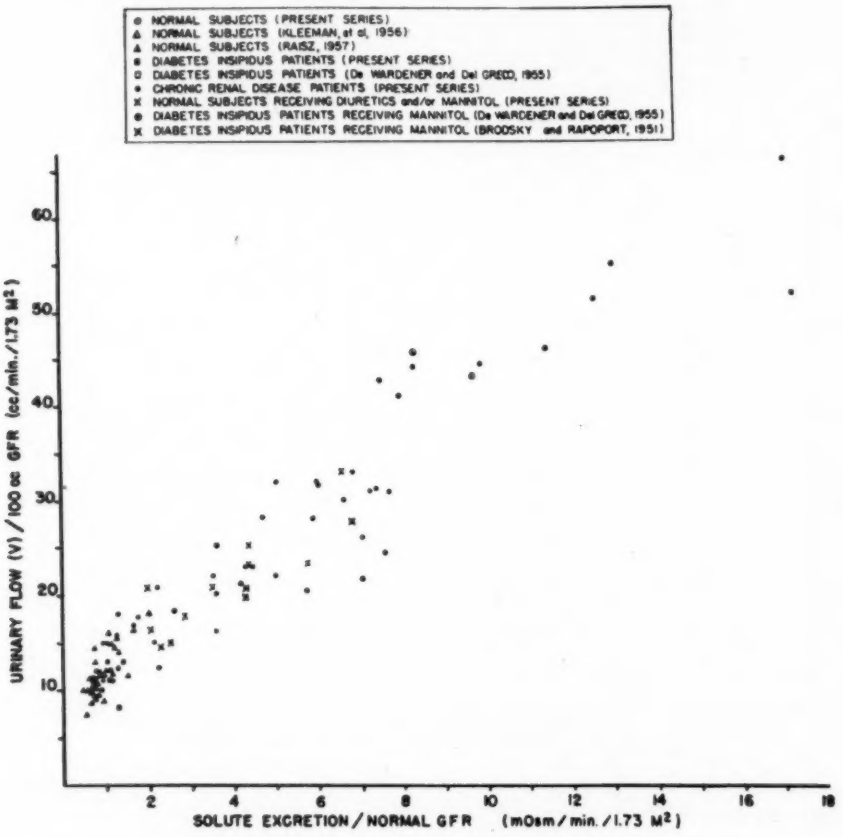

Fig. 4. (Derived data). Effect of the rate of solute excretion on the maximal urinary flow per 100 ml. of glomerular filtrate.

Within the limits of the methods used in the present study, the maximal water diuresis in chronic renal disease is normal for the mass of residual nephrons. This substantiates the observations and interpretation of Bricker, and associates,[22] who studied dogs with experimental renal failure, and of Walser and Orloff,[9] in their study of nephrotic patients. Further evidence for a normal mechanism for urinary dilution is the observation that, as in normal subjects, a significant reduction in solute excretion in chronic renal disease *decreases* the minimal urinary osmolality (improves the diluting capacity).[23]

In Fig. 7, the maximal urinary osmolality during the Pitressin test in patients with renal disease is plotted against the "corrected" rate of solute

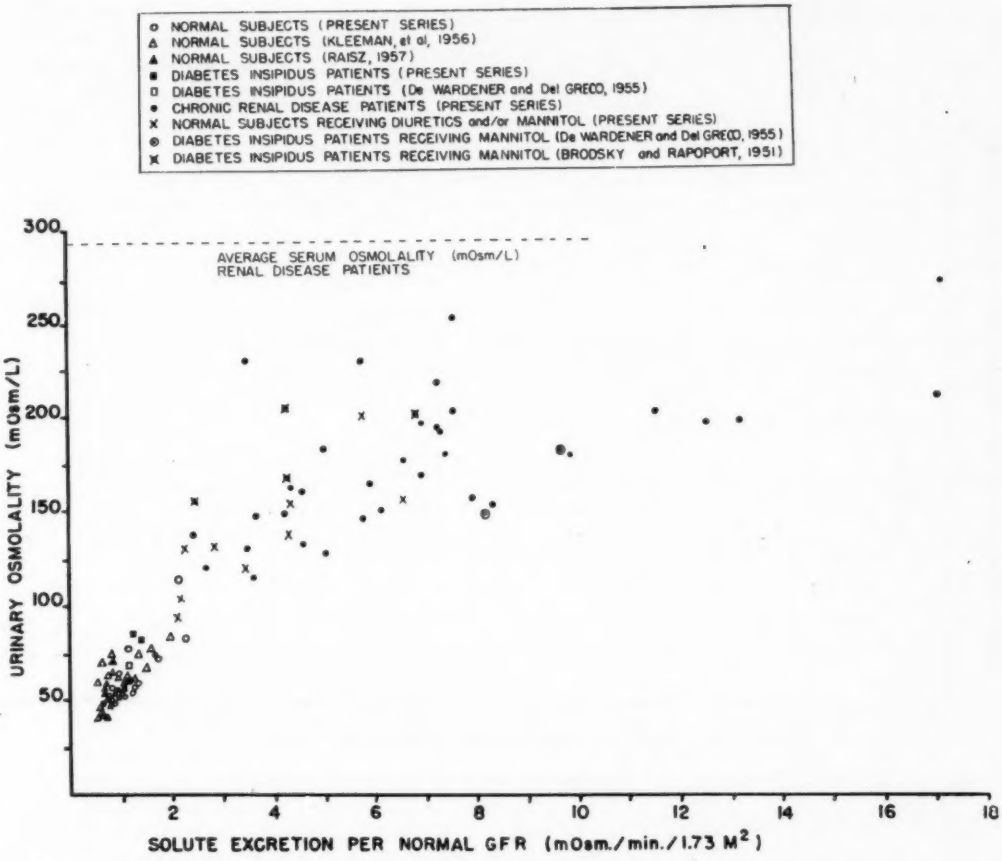

Fig. 5. (Derived data). Effect of the rate of solute excretion on the minimal urinary osmolality.

excretion; these are represented by the points in the figure. Superimposed is a curve representing the average data from hydropenic normal subjects at comparable rates of solute excretion.[2, 6, 24, 26, 27] This strongly suggests that solute diuresis per se cannot for the defect in concentrating capacity present in chronic renal disease when it is expressed as maximal urinary osmolality attained at any predicted rate of solute excretion. This confirms the observations of Baldwin and co-workers,[5] Dorhout-Mees,[25, 26] and the recent report of Franklin and associates.[28] However, the other parameter of concentrating capacity, the maximal rate of tubular reabsorption of free water in milliliters per minute ($T_MC_{H_2O}$) could not be calculated reasonably from the given data, and no statement about this parameter can be made.

Addendum. Since completion of these studies we have encountered our first example of a true defect in urinary dilution. The patient, a 50-year-old man with bilateral atrophic pyelonephritis, was unable to dilute the urine below the osmolality of the simultaneously collected serum. This was confirmed on 2 occasions. During a maximal sustained water load the renal hemodynamics and parameters of water diuresis in the patient were as follows:

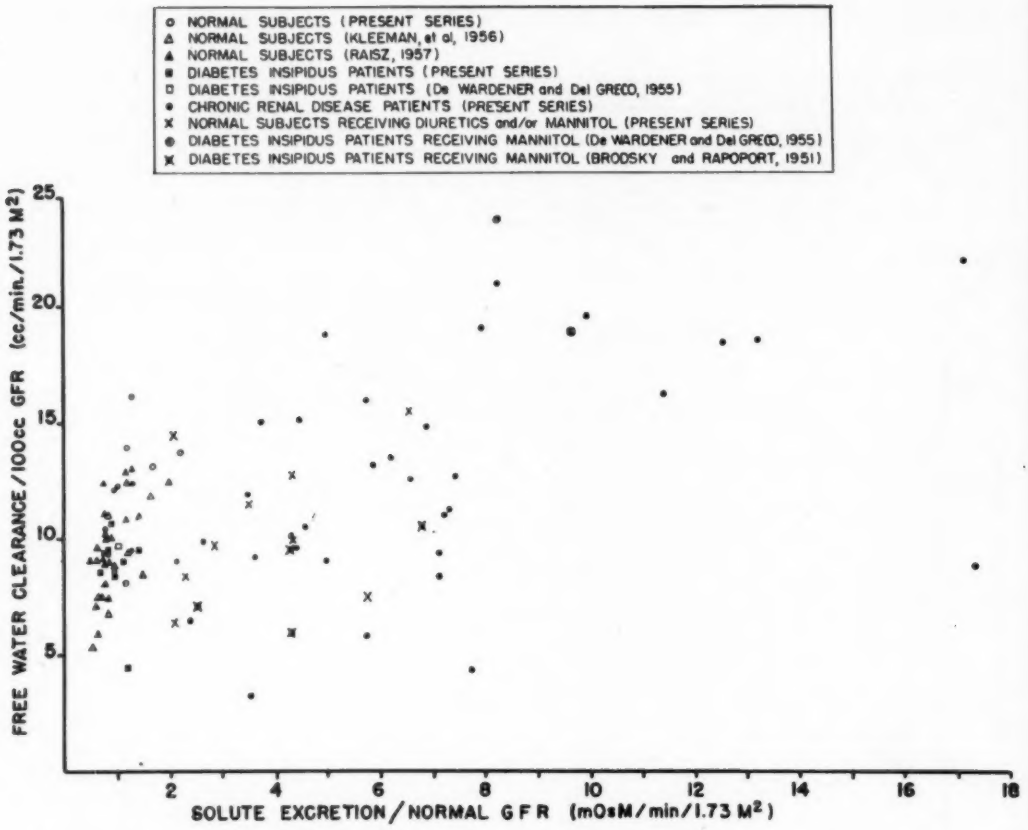

Fig. 6. (Derived data). Effect of the rate of solute excretion on the water clearance (C_{H_2O}) per 100 ml. of glomerular filtrate.

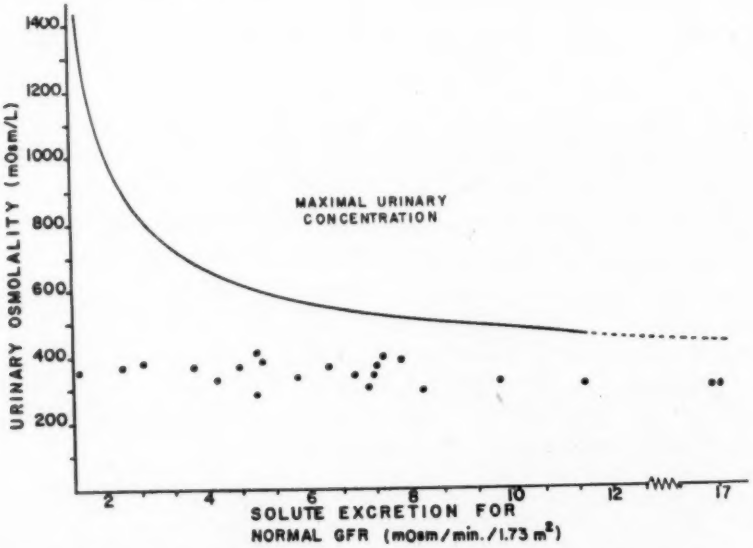

Fig. 7. Concentrating capacity of patients with renal disease compared with capacity of normal subjects.

PAH clearance, inulin clearance, maximal urinary flow, minimal urinary osmolality, and plasma osmolality were 36, 8, and 1.4 milliliters per minute, 305, and 306 mOsm. per kilogram, respectively. It is of extreme interest that this case was also a typical example of "salt-losing nephritis." The concentration of sodium in urine did not fall below 95 mEq. per liter, despite 4 days of rigid salt restriction (200 mg. per day) and simultaneous administration of 2 mg. per day of 9-alpha-fluorohydrocortisone. It is most probable that the major defect in sodium conservation was in the distal segment of the nephron, that area responsible for creating a maximally dilute urine during a sustained water diuresis.

Grateful acknowledgement is made of the assistance of Joanne Wilson and Trudy Isaacs.

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An evaluation of maximal water diuresis in chronic renal disease.

II. Effect of variations in sodium intake and excretion

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WITH THE TECHNICAL ASSISTANCE OF GARY LUNDE and ROSE TWISS

In a previous paper we concluded that a true defect in maximal urinary dilution did not exist in chronic renal disease. The observed values seemed adequately explained by the relative osmotic diuresis in each of the residual nephrons. However, the defect in maximal concentrating capacity was consistently greater than anticipated from the osmotic load per nephron. If these observations and conclusions are correct, a reduction in solute load per nephron should improve the maximal diluting capacity without appreciably correcting the concentrating defect. In the present study the rate of solute excretion was decreased by marked reduction of the salt content of the diet for 3 days prior to the sustained water load. This diet change, and the reduction in solute excretion, caused a consistent improvement in the maximal diluting capacity without improving the defect in maximal concentrating capacity. These findings are consistent with the suggestion that diluting capacity is normal in the residual nephrons in chronic renal disease, but that a true defect in concentrating capacity exists.

The previous report[1] suggests that the apparent defect in urinary dilution in patients with chronic renal disease can be explained by an increased solute

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Supported by The National Heart Institute, United States Public Health Service Grant No. A-1634.

Received for publication June 5, 1960.

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load within the residual nephrons. The minimal urinary osmolalities of patients with renal insufficiency were found to be approximately equal to those of normal subjects during maximal water diuresis at *comparable solute loads.*[1] In contrast, a true concentrating defect may be present in chronic renal disease. If, as postulated, no "true" impairment of diluting ability is present, a reduced rate of solute excretion should result in a more dilute urine (decreased minimal osmolality).[2]

Accordingly, in the present study, solute excretion was reduced by restriction of sodium intake.

Materials and methods

Sixteen paired studies were performed on 15 male azotemic patients with stable chronic renal disease who were selected by the criteria previously outlined.[1] The diagnoses are listed in the first column of Table I.

Diets and sodium intake. Each patient was placed on 2 separate diets (A and B), differing only in their sodium content, for 5 to 7 days prior to and during the test period. In 12 instances, diet A was given first; 4 patients (Nos. 2, 5, 8, and 14) received diet B first.

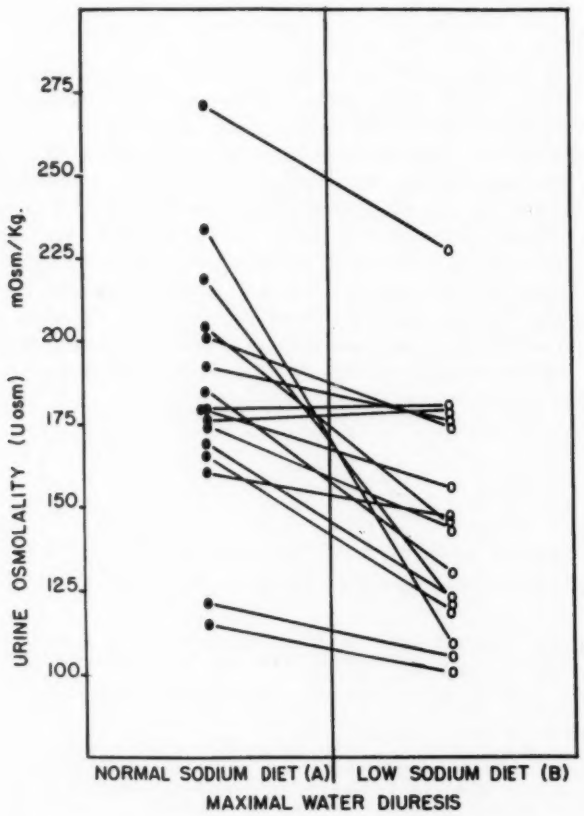

Fig. 1. Effect of normal *(A)* and low *(B)* sodium intake on the renal diluting ability (minimal urinary osmolality) during maximal water diuresis. Each line interconnects the results obtained for each patient on each diet.

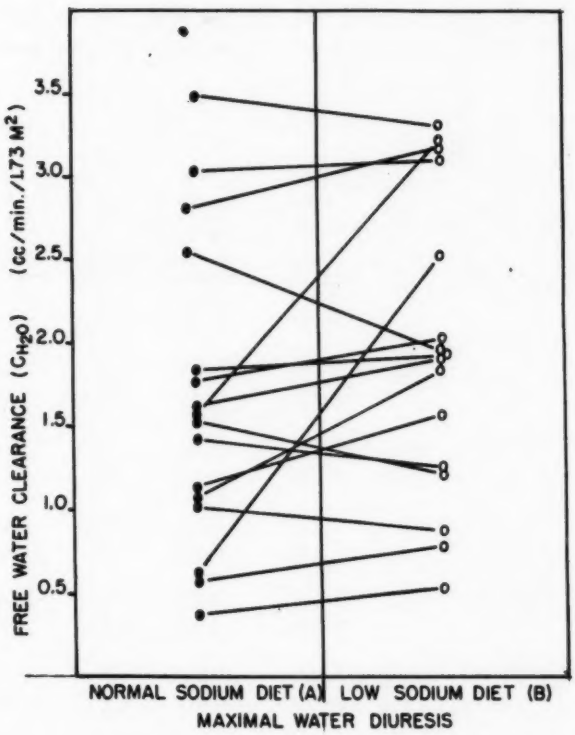

Fig. 2. Effect of normal *(A)* and low *(B)* sodium intake on the free water clearance *(C_{H_2O})* during maximal water diuresis.

DIET A (NORMAL SODIUM DIET). Diet A contained approximately 60 to 80 Gm. of protein and 90 to 175 mEq. of sodium daily.

DIET B (LOW SODIUM DIET). Diet B had the same protein content as diet A, but only 10 mEq. of sodium daily.

Distilled water was given ad libitum. The diets were approximately isocaloric. While on the low sodium diet, 3 patients (Nos. 2, 3, and 4) received a potent mineralocorticoid (MC), 9-alpha fluoroprednisolone, 0.25 mg. twice a day on the day preceding and the day of testing. The contents of the diets are listed in Table I.

Dilution and concentration tests. The procedures described in the previous report[1] were used to study the diluting and concentrating ability of all patients. The concentration of urea in the serum and urine was measured by the method of Archibald and associates.[3]

A timed 24 hour urine collection was made while each patient was on each diet in order to measure total volume and sodium excretion.

All observed data during water diuresis are corrected to 1.73 M^2 surface area. The derived rate of solute excretion per normal renal mass ($\frac{U_{osm}V}{C_{in}} \times C_t$) was calculated as in the previous paper.[1] $U_{osm}V$ and C_{in} represent solute excretion and inulin clearance, respectively.

Results

I. Sustained water diuresis (Table I and Figs. 1 through 5).

URINARY OSMOLALITY, C_{H_2O} AND SOLUTE EXCRETION. The minimal urinary osmolality during a sustained water diuresis was significantly lowered by sodium

Table I. Water diuresis in chronic renal disease patients on normal (A) and low (B) sodium

Patient	*Diagnosis*	*S.A. (M^2)*	*Diet (Na-mEq./day)*	*Serum creatinine (mg. %)*	*Urinary flow (ml./minute)*	*C_{in} (ml./minute)*	*C_{pah} (ml./minute)*	*Urine osmolality (mOsm./Kg.)*	*Solute excretion (mOsm/minute)*
1. H. C.	Chronic pyelonephritis	2.03	A-100	11.0	2.4	11.3	----	2,341	.571
			B- 10	14.3	1.3	7.8	----	109	.140
2. P. W.	Nephrosclerosis	1.59	B- 10	4.9	2.7	7.8	40.3	156	.418
			A-175	4.8	3.6	10.9	73.0	179	.653
3. L. E.	Chronic glomerulonephritis	1.83	A-175	3.4	2.4	19.0	93.1	218	.519
			B- 10	3.2	4.6	25.5	120.7	122	.564
4. C. W.	Nephrosclerosis	1.78	A-100	8.3	3.8	8.5	22.8	179	.678
			B- 10	8.9	4.5	10.1	21.3	181	.816
5. F. B.	Chronic glomerulonephritis	2.02	B- 10	5.2	3.0	13.9	135.0	145	.302
			A-100	4.5	3.2	14.6	136.0	204	.312
6. W. B.	Malignant nephrosclerosis	1.84	A- 90	5.8	2.4	11.9	48.1	169	.407
			B- 10	5.6	3.1	11.5	42.5	123	.383
7. W. H.	Chronic pyelonephritis	1.82	A- 90	18.1	2.3	4.5	8.8	272	.631
			B- 10	22.8	1.8	3.9	8.3	227	.415
8. P. D.	Nephrosclerosis	2.09	B- 10	10.1	2.0	6.9	20.9	174	.352
			A-100	10.0	2.7	7.9	29.6	202	.553
9A. L. C.	Chronic pyelonephritis	1.81	A-100	3.5	2.9	11.6	53.8	115	.338
			B- 10	3.4	3.2	13.4	68.2	102	.322
9B. L. C.			A-175	4.2	6.7	22.5	52.0	165	1.106
			B- 10	4.8	6.0	19.6	59.8	146	.876
10. G. T.	Malignant nephrosclerosis	2.03	A-175	7.4	3.6	11.8	63.3	176	.632
	Chronic pyelonephritis		B- 10	9.7	3.0	10.0	45.3	179	.539
11. R. K.	Chronic pyelonephritis	1.57	A-175	5.2	4.0	15.4	51.7	185	.750
			B- 10	5.7	5.8	14.7	68.2	130	.750
12. J. C.	Polycystic kidneys	1.62	A-175	2.5	6.2	24.5	87.4	122	.754
			B- 10	2.7	5.1	21.2	85.6	106	.544
13. J. D.	Chronic glomerulonephritis	1.81	A-175	4.6	6.2	17.1	74.1	174	1.082
			B- 10	5.1	4.0	12.1	58.8	145	.573
14. J. M.	Acute glomerulonephritis	1.83	B- 10	2.9	5.3	29.7	217.0	120	.638
			A-175	2.8	6.5	25.2	182.0	166	1.072
15. W. G.	Chronic glomerulonephritis	1.83	A-175	8.0	4.6	8.5	49.7	193	.886
			B- 10	8.5	4.4	8.5	55.0	177	.770

sodium diets

Solute excretion (mOsm./minute)	*Serum osmolality (mOsm./Kg.)*	C_{osm} *(ml./minute)*	C_{H_2O} *(ml./minute)*	U_{Na} *(mEq./liter)*	$U_{Na}V$ *(μEq./minute)*	*Serum Na (mEq./liter)*	C_{H_2O} *100 ml. GFR (ml./minute)*	*Predicted solute excretion Normal* C_{in} *(mOsm./minute)*	*24 hour urine Na excretion volume*	
									(mEq.)	*(ml.)*
.571	303	1.9	0.5	34.5	84.2	----	4.9	6.3	142.0	4,130
.140	286	0.5	0.8	2.1	2.7	----	10.3	1.5	6.0	2,465
.418	291	1.4	1.3	15.6	41.8	129.3	15.9	4.8	17.2	1,243
.653	292	2.2	1.4	46.7	170.5	131.2	12.9	7.4	47.9	1,560
.519	292	1.8	0.6	32.6	77.6	139.0	3.2	3.4	----	----
.564	268	2.1	2.5	12.0	55.4	138.5	9.9	2.7	----	----
.678	312	2.2	1.6	30.6	116.0	134.7	19.1	9.9	50.0	1,960
.816	313	2.6	1.9	27.7	125.0	133.8	18.8	10.0	22.5	1,600
.302	436	1.4	1.6	10.6	31.9	140.2	11.3	3.7	11.2	1,545
.312	659	2.1	1.1	39.9	128.9	145.0	7.7	5.6	29.5	1,295
.407	300	1.3	1.1	9.8	23.6	135.0	9.0	4.2	24.8	2,335
.383	300	1.3	1.8	5.7	17.7	137.4	16.0	4.0	10.9	3,035
.631	325	1.9	0.4	67.2	144.0	137.0	8.4	17.4	202.0	3,015
.415	321	1.3	0.5	47.1	86.0	134.0	13.8	11.4	96.2	2,015
.352	308	1.1	0.9	14.9	30.1	138.4	12.8	5.5	22.3	2,080
.553	318	1.8	0.9	34.4	94.3	143.6	12.6	8.7	48.0	1,375
.338	288	1.2	1.7	20.1	59.0	134.7	15.3	3.6	80.6	2,920
.322	286	1.1	2.1	17.1	54.0	133.8	15.1	3.3	55.3	2,625
1.106	302	3.7	3.0	49.2	330.0	137.6	13.5	6.1	288.0	4,100
.876	302	2.9	3.1	30.8	185.0	126.4	15.8	4.8	160.0	2,675
.632	304	2.1	1.5	46.7	167.7	143.3	12.8	6.6	----	----
.539	303	1.8	1.2	40.1	120.7	141.7	12.3	5.7	79.4	1,430
.750	303	2.5	1.5	41.5	168.0	138.8	10.2	6.0	140.0	2,620
.750	295	2.5	3.3	23.6	136.0	134.6	21.9	6.0	104.5	3,520
.754	280	2.7	3.5	28.0	173.0	135.2	14.2	3.1	104.5	3,520
.544	283	1.9	3.2	11.2	57.0	132.3	15.7	2.8	28.2	3,895
1.082	293	3.7	2.5	50.3	312.9	138.8	14.8	7.8	28.4	870
.573	285	2.0	2.0	28.7	113.4	137.5	16.0	4.1	53.8	1,830
.638	297	2.2	3.1	12.4	65.9	143.4	10.7	2.7	20.7	2,760
1.072	293	3.7	2.8	36.0	232.6	145.6	11.1	5.3	184.3	2,865
.886	321	2.8	1.8	43.1	117.8	135.2	21.5	12.9	54.6	2,120
.770	317	2.4	2.0	38.4	167.0	133.0	22.6	11.2	70.3	2,130

Table II. Vasopressin (Pitressin) concentration test in chronic renal disease patients on normal (A) and low (B) sodium diets

Patient	*Diet*	*Serum creatinine (mg. %)*	*Volume (ml./ minute)*	*Urine osmolality (mOsm./ Kg.)*	*Serum osmolality (mOsm./ Kg.)*	$U_{OSM.}$ / $P_{OSM.}$	U_{UREA} *(mM./ liter)*	$U_{UREA}V$ *(μM/) minute)*
1. H. C.*	A	11.0	1.8	294	316	0.9	85.0	154.0
	B	14.3	2.0	230	305	0.8	69.0	138.0
2. P. W.	B	4.8	1.7	302	290	1.0	38.3	64.0
	A	4.9	2.5	292	293	1.0	33.3	84.6
3. L. E.	A	3.4	1.3	421	294	1.4	61.3	79.7
	B	3.2	1.3	383	287	1.3	106.7	138.7
4. C. W.	A	8.3	1.8	322	313	1.0	62.3	106.0
	B	8.9	2.3	235	317	0.7	45.8	103.5
5. F. B.	B	5.2	2.0	385	304	1.3	89.0	180.7
	A	4.5	2.5	383	308	1.2	63.3	158.0
6. W. B.	A	5.8	1.8	330	310	1.1	85.0	154.0
	B	5.6	2.0	345	302	1.1	69.0	138.0
7. W. H.	A	18.1	---	281	325	0.9	---	----
	B	22.8	---	---	---	---	---	----
8. P. D.	B	10.1	1.8	366	310	1.2	95.0	167.2
	A	10.0	2.6	393	322	1.2	93.8	243.9
9A. L. C.	A	3.5	2.6	371	291	1.3	---	----
	B	3.4	1.7	401	292	1.4	64.5	103.8
9B. L. C.	A	4.2	1.0	394	307	1.3	56.7	56.7
	B	4.8	2.7	386	305	1.3	70.0	187.0
10. G. T.	A	7.4	1.4	369	306	1.2	63.0	75.0
	B	9.7	1.3	372	307	1.2	86.0	92.9
11. R. K.	A	5.2	1.6	420	302	1.4	71.0	114.0
	B	5.7	1.1	425	305	1.4	95.3	106.0
12. J. C.	A	2.5	1.9	358	288	1.2	64.0	123.0
	B	2.7	0.8	331	284	1.2	79.0	63.2
13. J. D.	A	4.6	2.8	351	298	1.2	64.6	182.8
	B	5.1	3.3	330	288	1.2	66.6	216.5
14. J. M.	B	2.9	2.3	373	299	1.2	96.6	225.1
	A	2.8	2.4	372	299	1.2	56.0	136.1
15. W. G.	A	8.0	---	---	---	---	---	----
	B	8.5	1.7	314	320	1.0	60.0	100.0

*This patient was maintained on each diet for several weeks before testing. The data obtained were from an average of 6 hour urine collection periods while the patient received aqueous Pitressin 0.3 ml. (6 units) subcutaneously every 4 hours for 24 hours.

restriction (diet B) in 14 of the 16 studies (Fig. 1). In two (Nos. 4 and 10) there was no change. The free water clearance (C_{H_2O}) was *increased* in all but 5 of the patients (Nos. 2, 8, 10, 12, and 13) following diet B (Fig. 2). The observed rate of solute excretion ($U_{osm}V$) (Table I) was reduced in all but 2 patients (Nos. 4 and 11) following sodium restriction.

The free water clearance per 100 ml. of GFR $\left(\frac{C_{H_2O}}{C_{in}} \times 100\right)$ *increased* as solute excretion corrected to normal GFR (approximate normal renal mass),[1] *decreased* during sodium restriction in 12 of the 16 experiments (Fig. 3). The magnitude of the change in free water clearance per 100 ml. GFR was variable and no constant correlation to the magnitude of the fall in solute excretion could be made.

SODIUM EXCRETION. While on diet B the concentration of sodium in the urine (Fig. 4, lower) and the rate of sodium excretion ($U_{Na}V$) (Fig. 4, upper) decreased in every instance except one (No. 4) during sustained water diuresis. However, the reductions in Patients Nos. 9A, 10, and 15 are of questionable magnitude.

Therefore, varying, but generally favorable, degrees of sodium conservation, after sodium restriction, were attained during water diuresis in the patients studied. Only 9 patients (Nos. 1, 2, 3, 5, 6, 8, 9A, 12, and 14) were able to reduce the urinary sodium concentration to under 20 mEq. per liter after the low sodium diet (Table I). Although the relationship was not constant, certain

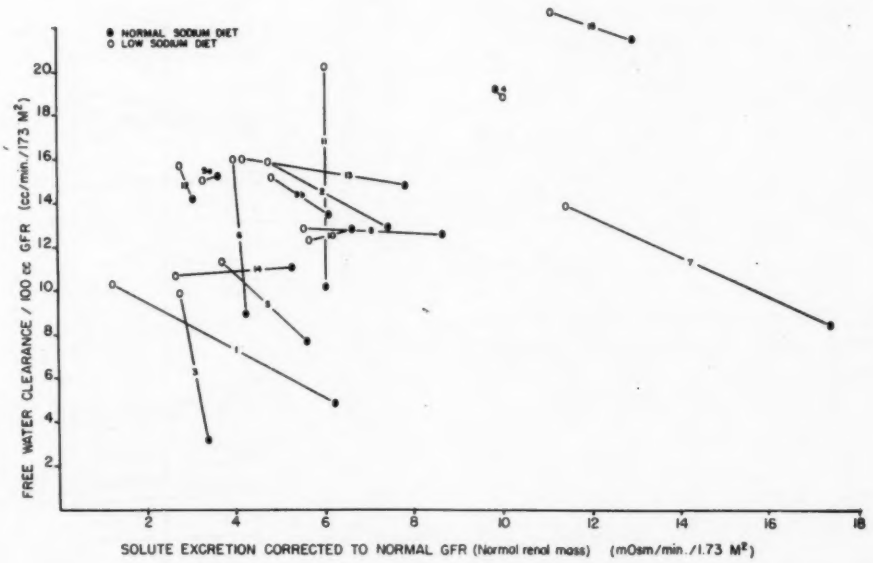
● NORMAL SODIUM DIET
○ LOW SODIUM DIET
FREE WATER CLEARANCE / 100 cc GFR (cc/min./1.73 M²)
SOLUTE EXCRETION CORRECTED TO NORMAL GFR (Normal renal mass) (mOsm/min./1.73 M²)

Fig. 3. (Derived data). The effect of variations in solute excretion on the free water clearance (C_{H_2O}) per 100 ml. GFR in each patient during maximal water diuresis. (The numbers refer to patients as listed in Table I).

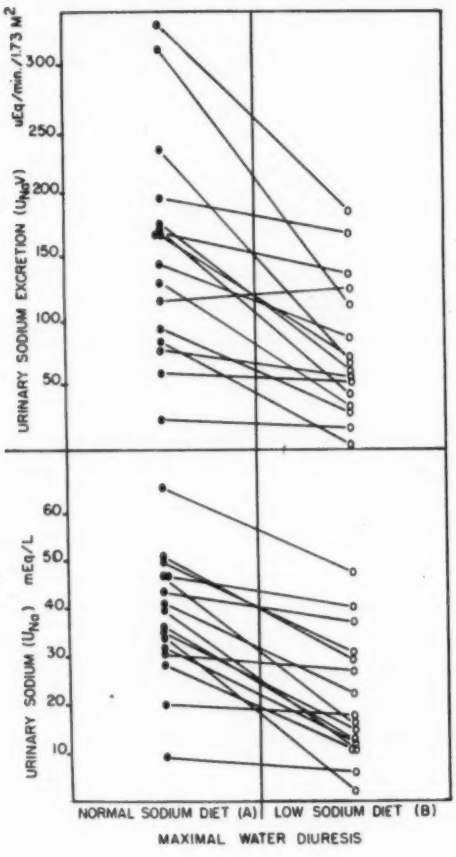

Fig. 4.

Fig. 4. Effect of normal (*A*) and low (*B*) sodium intake on urinary sodium concentration (lower) and excretion (upper) during maximal water diuresis.

Fig. 5. Effect of normal (*A*) and low (*B*) sodium intake on glomerular filtration rate (C_{in}) and renal plasma flow (C_{pah}) during maximal water diuresis.

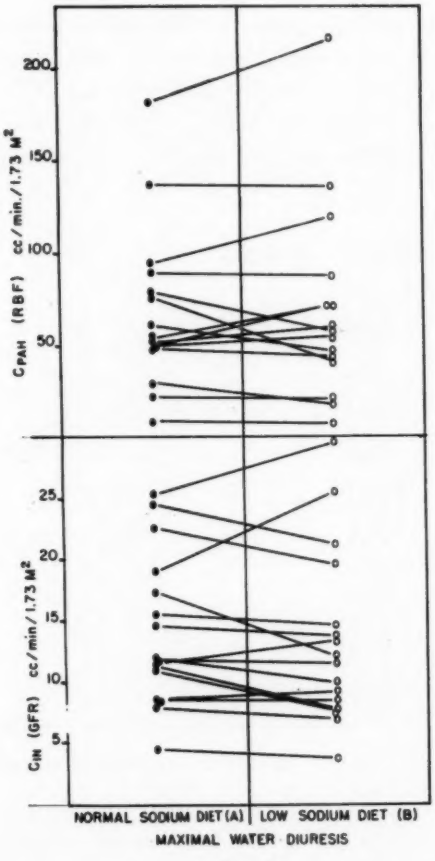

Fig. 5.

cases demonstrating the poorest ability to reduce sodium excretion also had the poorest reduction in urinary osmolality following salt deprivation (e.g., Nos. 4, 10, and 15).

SERUM SODIUM. The serum sodium fell 1 to 3 mEq. per liter in most instances, but dropped 4.8 mEq. per liter twice (Nos. 5 and 8) and 11.2 mEq. per liter once (No. 9B) following sodium restriction (Table I).

INULIN AND PAH CLEARANCES. Moderate decreases in inulin clearance (GFR) occurred in 5 patients (Nos. 1, 2, 9B, 12, and 13) with little change or slight increases in the others after salt restriction (Fig. 5, lower). Despite the fall in GFR, these individuals still demonstrated an *augmentation* of their diluting ability. A reduction in GFR would be expected to impair maximal urinary dilution.[4, 5]

Similar patterns were observed in PAH clearances (RBF) (Fig. 5, upper).

II. Maximal urinary concentration (Table II and Fig. 6). There was no consistent change in maximal urinary concentration (vasopressin test) or osmotic urine-to-plasma (U/P) ratio following sodium restriction (Fig. 6).

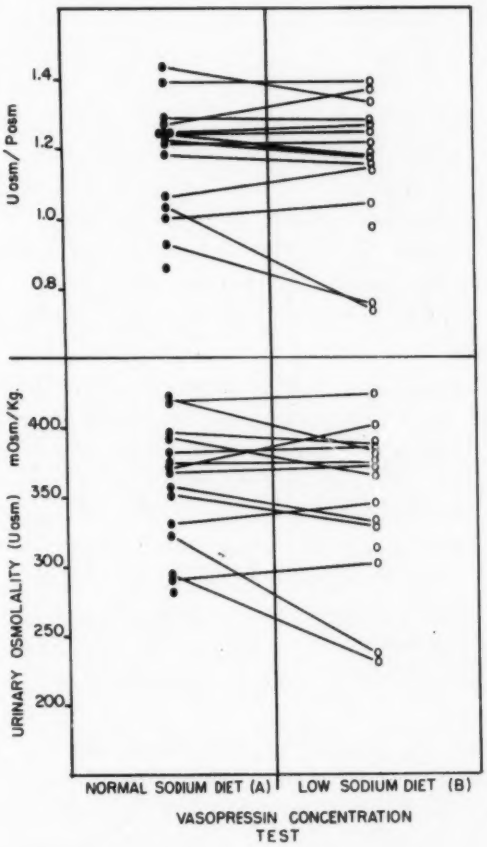

Fig. 6. Effect of normal *(A)* and low *(B)* sodium intake on concentrating ability during vasopressin infusion (2 patients were tested on only one of the diets).

Variations in the excretion of urea in normal subjects significantly alter maximal concentrating ability.[6] In the present study, urea excretion showed no consistent change (Table II) and could not be correlated with the maximal osmolality of the urine.

III. Twenty-four hour excretion of sodium (Table I). Sodium restriction resulted in a significant reduction in the 24 hour excretion of this ion in 13 of the 14 patients in whom it was measured. In 3 patients (Nos. 1, 5, and 6) sodium excretion was equal to or less than intake, and 1.5 to 2 times intake in 4 others (Nos. 2, 4, 8, and 14). More prolonged sodium deprivation may have reduced these excretion rates further.[7]

Although Patients Nos. 4, 6, and 9A showed little change or even greater sodium excretion per minute after diet B during maximal water diuresis, examination of the 24 hour sodium excretion rates demonstrates a decided ability to reduce urine sodium after sodium restriction.

Discussion

In this study, by restricting sodium intake in patients with chronic renal disease, a reduction in the minimal urinary osmolality during water diuresis was attainable. Where the urinary osmolality showed no significant fall, sodium and/or total solute excretion was not notably altered. It has been demonstrated that patients with chronic renal disease exhibit varying degrees of sodium excretion and conservation following sodium deprivation.[7-9] In the present study this was similarly observed. A few patients showed little or no fall in sodium excretion after the low sodium diet *during water diuresis.* However, with the exception of Patient No. 15, the 24 hour rate of sodium excretion, when measured, was considerably lower after sodium restriction.* This may indicate that a relative augmentation of sodium excretion by a water diuresis is possible in chronic renal disease.

Free water clearance (C_{H_2O}), a measurement of the amount of solute-free water excreted during the diluting process, increases in normal subjects[1, 2, 11] or diabetes insipidus patients[12, 13] subjected to an osmotic load superimposed on a maximal water diuresis. This implies that the diluting segment of the nephron in normal subjects is capable of reabsorbing a greater proportion of the solute in the increased volume of isosmotic tubular fluid delivered to it. In the previous report,[1] for the group of chronic renal disease patients studied, the C_{H_2O} per 100 ml. GFR was usually highest in those patients with the greatest predicted solute excretion, up to approximately 8 mOsm. per minute per 1.73M^2. Higher predicted rates of solute excretion were not associated with greater free water clearances.

In this paired study, in contrast to the normal, the C_{H_2O} per 100 ml. GFR was *increased* or not significantly changed as the predicted solute excretion was *reduced* by a restricted sodium intake. Possible causes for this enhanced free water clearance after sodium deprivation in chronic renal disease include:

*The explanation for the discrepancy in this one patient may have been due to his taking salt without our knowledge or, more likely, he may be a true "salt-losing" nephrictic.[10]

A decreased back-diffusion of water in the diluting segment of the residual nephrons as a consequence of variations in circulating antidiuretic hormone (ADH).

An increased reabsorption of sodium in the diluting segment of the nephrons without accompanying back-diffusion of water.

It is assumed that ADH release from the neurohypophysis is inhibited by a large water load as completely in chronic renal disease patients as it is in normal subjects. In the absence of ADH, the normal diluting segment is virtually impermeable to water, although movement of small amounts of water is probably not completely restricted.[14] It might be considered that circulating ADH was not completely absent during water diuresis in the patients on the normal salt intake, while it was absent in those on the restricted sodium diet. This could account for the slight increase in C_{H_2O} observed during the low sodium diet. However, since salt deprivation in patients with renal insufficiency often results in a contraction of the extracellular volume, a potent stimulus to ADH release,[15] it seems an unlikely hypothesis. Therefore, assuming that ADH is absent on both diets during the sustained water load, the difference in C_{H_2O} probably cannot be explained by variations in circulating ADH.

An increased reabsorption of sodium in the diluting segment of the nephrons accompanying back-diffusion of water could account for a rising C_{H_2O} after sodium restriction. It has been shown that the per cent excretion of sodium falls[8] and that tubular reabsorption of sodium increases[9] in chronic renal disease patients after salt deprivation. To make the urine more hypotonic with a *greater* C_{H_2O} despite a fall in sodium excretion, requires that a larger *proportion* of sodium be reabsorbed in the distal portion of the residual nephrons than was reabsorbed in this site while the patient was on the normal salt intake diet. This suggests that reducing the solute load to the distal tubules in chronic renal disease may improve the ability to reabsorb sodium without water in these segments.

Lowering the sodium intake and excretion had little influence in improving the concentrating ability of the patients studied. Franklin and associates[16] made similar observations, that lowering the osmolar clearance by hemodialysis did not improve the osmotic U/P ratio in patients with renal insufficiency. Baldwin and co-workers,[17] by calculating the ratio $\frac{T_M C_{H_2O}}{C_{in}}$ (the quantity of solute free water required to restore a hypertonic urine to an isosmotic state with respect to GFR), found that there was a specific defect in the concentrating mechanism in the majority of their patients with renal failure.

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The relation between glomerular function and histology in acute glomerulonephritis

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Twenty-one individuals with acute glomerulonephritis have been studied by means of renal biopsy and inulin and para-aminohippurate clearances. Nine of these were studied more than once. Of the observations made, the degree of glomerular cellularity and the inulin clearance seemed to correlate best. The ratio of glomerular to tubular function seemed to give some indication of the progress of the disease in that renal histology improved in those patients whose ratio rose during the course of the disease but remained unchanged in those whose ratio remained low. As has been observed in previous studies, the less the reduction in renal function during the acute stage of the disease the more rapid the return of function to normal. However, functional and histologic changes during the early phase of the disease may be extensively deranged without necessarily indicating a poor prognosis.

Patients with acute glomerulonephritis demonstrate varying degrees of depression of glomerular function during the acute phase of the disease and gradual improvement in function as healing occurs. That this depression may be related to the degree of pathologic involvement of the kidney has been suggested by previous work.[1-4] In order to further test this suggestion the following investigation was undertaken.

Methods

Renal needle biopsy was performed in each case in which the diagnosis of acute glomerulonephritis was entertained as soon as possible after the tentative diagnosis was made. Biopsy was followed in 2 or 3 days by the measurement of renal functions. Renal biopsies were performed as previously reported.[5] Glomerular function (GFR) was estimated

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Supported in part by United States Public Health Service Grants H-3732 and A-2234, The Washington Heart Association, and the Meyer Foundation.

Received for publication Jan. 5, 1961.

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Table I. Pathologic findings in patients with glomerulonephritis

Patient No.	Date	Time from onset*	Glomerular filtration rate (ml./minute)	Effective renal plasma flow (ml./minute)	Tubular maximum PAH (mg./minute)	Filtration fraction	Glomerular filtration rate/tubular maximum PAH	ERPF/tubular maximum PAH	Glomerular changes†		
									Cellularity	Impaired vascularity	Basement-membrane thickening
1	4/1/52	16	17	54		.31			4	3	3
	4/11/52	25	34	96	18	.36	1.89	5.33	3	2	2
	1/27/55	3 years	95	508	84	.19	1.13	6.05	1	0	1
2	10/5/53	39	109	366	59	.32	1.85	6.20	2	0	1
3	3/1/54	13	74	109	75	.69	0.98	1.45	3	3	2
	4/2/54	44	72	890	84	.08	0.86	10.6	3	3	3
4	4/1/54	11	111	532	132	.21	0.84	4.03	2	1	2
5	8/26/57	11	67	348	69	.19	0.97	5.04	3	3	3
	9/13/57	28	76	609	92	.12	0.83	6.61	2	3	3
6	11/15/57	11	15	86	6	.18	2.50	14.3	1	2	1
	12/6/57	32	137	905	102	.15	1.34	8.86	1	0	1
7	12/13/57	5	52	498	96	.10	0.54	5.19	4	4	1
8	12/1/55	14 months	155	524	49	.30	3.16	10.7	1	1	2
9	2/21/55	32	91	657	108	.16	0.84	6.09	2	2	2
	3/31/55	73	128	506	49	.25	2.61	10.31	1	1	2
	10/18/55	270	95	372	96	.26	0.99	3.87	1	1	2
10	4/27/59	51	136	475	169	.28	0.80	2.80	2	0	0
11	8/23/54	42	58	390	40	.15	1.45	9.75	2	2	3
12	12/9/55	15 months	107	432	73	.25	1.47	5.90	2	1	2
13	1/16/59	9	106	562	168	.19	0.63	3.34	3	2	0
14	3/12/58	32	72	428	84	.17	0.86	5.09	2	2	2
	4/9/59	59	128	485	64	.26	2.00	7.58	1	0	1
15	4/8/58	15	82	686		.12			3	3	3
16	6/27/58	21	117	766	88	.15	1.33	8.71	2	3	3
17	3/19/56	128	54	401	21	.14	2.56	19.10	2	1	2
	4/12/56	153	39	278	30	.14	1.30	9.26	1	2	2
	6/8/56	209	24	198	36	.12	0.67	5.50	3	1	2
	7/25/56	256	44	350	13	.12	3.38	26.9	1	2	3
	1/24/57	406	4	18		.24			1	2	3
18	11/1/56	13	98	625	76	.16	1.29	8.22	1	1	1
	12/3/56	46	110	631	79	.17	1.39	8.00	2	2	3
19	11/16/56	40	63	455	78	.14	0.81	5.84	3	3	3
	12/6/56	63	73	464	97	.16	0.75	4.78	3	3	3
20	12/31/56	36	58	374	47	.16	1.24	7.96	3	3	3
21	5/9/57	14	110	610		.18			3	3	3

*Time expressed in days, except as indicated.
†Glomerular changes recorded as 0, no pathologic changes to 4+, maximum pathologic changes.

by measurement of the clearance of inulin. Sodium para-aminohippurate (PAH) excretion was used to estimate effective renal plasma flow (ERPH) and tubular function (TmPAH). Renal biopsies were performed as early in the disease as possible, 1 month later, and whenever practical thereafter. Renal functions were performed in the 12 hour postprandial patient who had received no more than 250 ml. of water 1 hour prior to the tests. Three or more clearance periods were carried out for each determination. Creatinine clearances done before and after biopsy in another group of patients showed no change, suggesting that renal biopsy has no effect on function.[6]

Renal biopsy specimens were fixed in Bouin's solution, paraffin embedded, sectioned, and stained with hemotoxylin, eosin, and by the periodic acid–Schiff technique. Sections were cut at a thickness of about 5 μ.

Results

Twenty-one patients were studied a total of 35 times. Twelve patients were studied once, 6 twice, and 3 were studied 3 or more times. The data are presented in Table I.

Normal values (above 100 milliliters per minute) for GFR were found in 4 patients on whom combined studies were done within the first 3 weeks of illness. None of these patients was very ill initially and all but one recovered without difficulty. Their renal biopsies showed minimal glomerular basement membrane thickening but a marked decrease in the apparent vascularity of the glomeruli and an increase in cellularity. A representative specimen is shown in Fig. 1, *A*.

Glomerular filtration could not be correlated with the time from onset of the disease, since the scatter of data made any over-all conclusion impossible except that each patient was different in the degree of injury and the rapidity of recovery.

In 9 patients serial determinations were possible. Seven of these patients showed some degree of improvement in their renal functions. One (Patient 3) showed improvement in ERPF but no change in GFR and Patient 9 had an initial improvement in GFR but a slow decrease in ERPF. The one patient showing a steady decrease in renal functions (Patient 17) gradually deteriorated over a year's time and died. The histologic findings in the renal biopsies in 4 of these patients seemed to indicate improvement, at least one patient (No. 9) showed some decrease in renal functions. Serial sections in this patient are illustrated. (Fig. 1, *B* through *D*)

Grading of renal pathology was estimated by 3 persons independently, as previously reported.[3] Grading was subdivided into degree of glomerular cellularity, basement membrane thickening, and ischemia. The grading of histologic changes was then plotted against renal function. The best correlation seemed to be that of inulin clearance against glomerular cellularity (Fig. 2), possibly because cellularity is most easily estimated, or because one of the major histologic disturbances seems to be an increase in the number of endothelial cells. Basement membrane thickening was observed to some extent in all patients. This was most marked in those patients who presented clinically with the nephrotic syndrome.[7] Histologically, these patients initially showed proliferative and exudative changes as well as thickening of the basement membranes.

*Time expressed in days, except as indicated.
†Glomerular changes recorded as 0, no pathologic changes to 4+, maximum pathologic changes.

These changes were associated with a clinical picture of edema, hypertension, hematuria, proteinuria, and normal serum cholesterol levels. The histologic picture then changed rapidly to resemble the more classical picture of basement membrane thickening seen in "nephrosis." Because of the initial histologic picture, however, these patients have been included.

"One plus" glomerular changes were often associated with inulin clearance values greater than 100 milliliter per minute, thus either "one plus" changes are not functionally significant or are in reality normal histologic findings. Inulin clearance values of 100 milliliter per minute or higher also were found in instances where histologic changes were graded as 2^+ and 3^+. GFR/TmPAH was

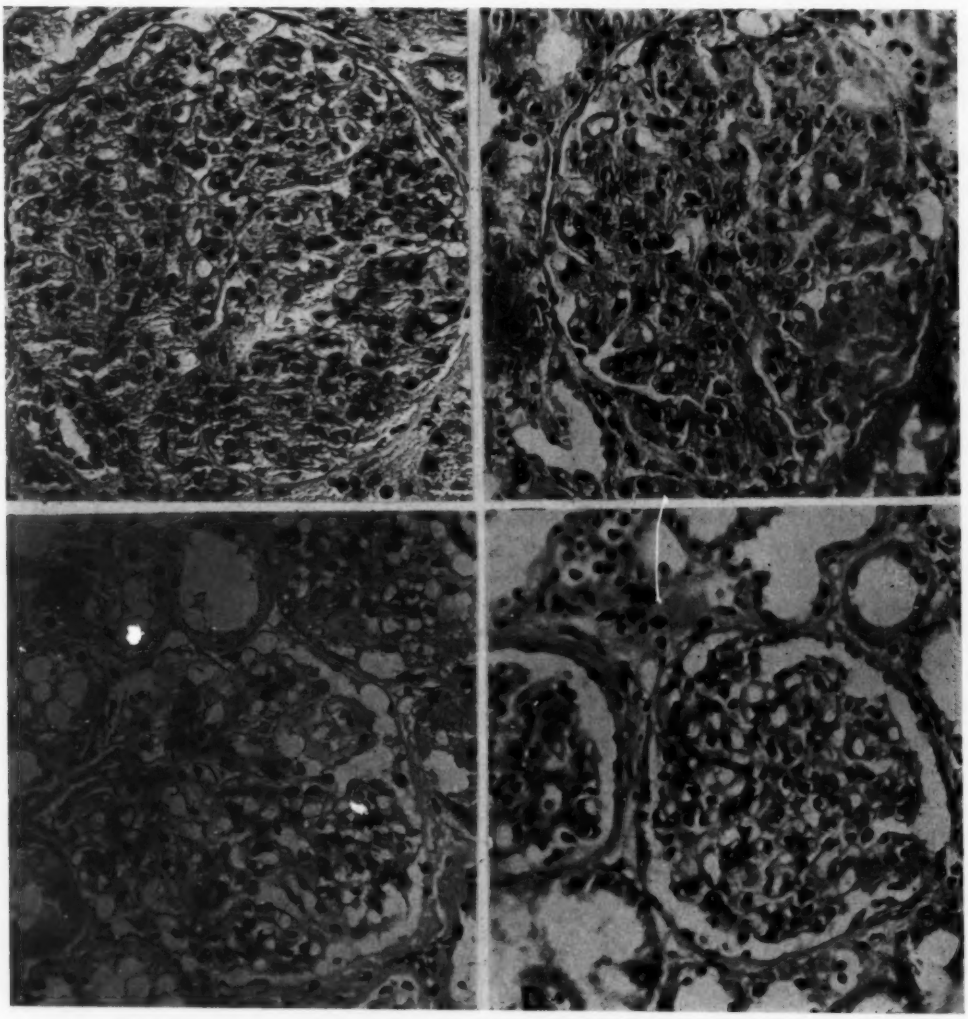

Fig. 1. A, Renal biopsy from a patient with minimal symptoms of acute glomerulonephritis showing slight basement membrane thickening, marked cellularity, and ischemia. *B, C, D*, Serial biopsies in a patient showing progression from an exudative-proliferative lesion to one primarily of basement membrane thickening. (Original magnification ×300.)

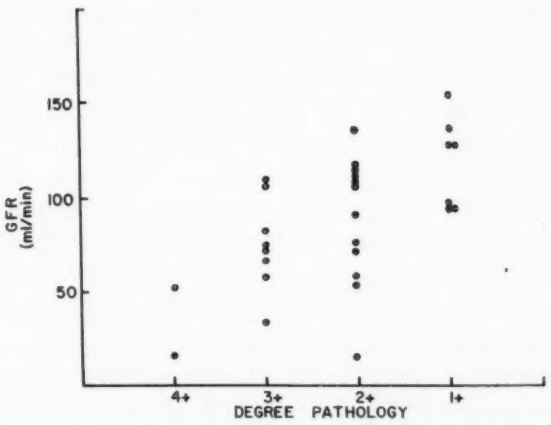

Fig. 2. The relation between glomerular cellularity and the glomerular filtration rate.

below 1.2 in 11 patients at some time during the course of the disease. This ratio was increased in 4 patients. The filtration fraction (GFR/ERPF) was high in 6, normal in 12, and low in 4 patients when done initially.

Discussion

Previous reports[1-4, 8] have indicated that renal function tests in acute glomerulonephritis give a reasonable indication of the state of renal histologic changes. Brod and Benešová[1] found agreement between endogenous creatinine clearance and glomerular changes in 15 out of 29 patients studied. Most of the lack of correlation was believed to be due to the presence of greater damage to the glomerulus than was indicated by measurements of the GFR. The present study indicates that inulin clearance gives a good estimation of glomerular damage although minimal ("one plus") changes histologically may not be reflected in altered glomerular filtration as measured by inulin clearance. It has also been pointed out[9] that an increased erythrocyte sedimentation rate may give a clue to the severity of the renal lesion.

Tubular changes in acute glomerulonephritis were the subject of a previous report.[10] It has been suggested that the ratio of inulin clearance to TmPAH (GFR/TmPAH) gives some idea of the relative status of glomerular versus tubular function. Assuming a normal range of about 1.2 to 2.0,[11] there were 11 patients who demonstrated a low GFR/TmPAH value at some time during their course, suggesting greater glomerular than tubular damage. Six patients with serial renal functions showed changes in this ratio during the course of the disease. In 4 patients (Nos. 1, 9, 14, 18) whose initial studies were made during approximately the first month of their disease, this ratio was low initially, then rose; in 2 (Patients 1 and 9) the ratio finally fell again. This did not occur in Patients 3, 5, or 19, whose values did not change appreciably. The ratio in Patients 6 and 17 fell during the disease process although it rose again prior to death in Patient 17. Earle and associates[2, 12] noted the low initial GFR/Tm ratio and the rise in this ratio as the disease progressed but did not describe the subsequent fall. It is interesting to note that the renal histology

improved in those patients whose values rose, but remained static in those in whom the ratio did not change. These findings, although based on a limited number of patients, suggest that glomerular damage predominates early in the course of glomerulonephritis, and is followed by the onset of tubular damage, which may temporarily exceed the degree of glomerular pathology, which in turn is followed by a healing of the tubular lesion. This seems to be confirmed histologically[10] and the maximum tubular disruption seems to have occurred by the end of 30 days. At this point tubular epithelial vacuolization, degeneration, and pseudodilatation reach their peak; thereafter they subside.

A reduced filtration fraction (GFR/ERPF) has been noted in previous studies[2, 12] even in the presence of hypertension. This was not true in our patients in whom values were variable. In those studied serially, 2 were high initially; 1 of these rose, then fell to normal, and the other fell markedly (Patient 3). The remainder of the values were normal to start and then increased in 3, decreased in 2, and remained unchanged in 2. In 1 patient (No. 17) who died, the value was normal initially, rose, then decreased, and finally just before death, returned toward the initial value. It would seem logical that variations in this value might be expected since it would depend on changes in the filtering membrane and patency of vascular channels. It would then be influenced by cellularity, fibrosis, exudation, and basement membrane thickening.

Previous reports [2, 4, 9, 12, 13] have found that the less the reduction in renal function the more rapid the return to normal occurred and that the initial degree of impairment bore an inconstant relation to the ultimate prognosis. In our patients, it is interesting to note, glomerular changes were still present after 3 years, even though clinical recovery had occurred.

Our results agree that, in general, patients who recovered from their acute attack clinically either had good renal function to start or improved rapidly after the onset of their disease. Deterioration of renal function is, as expected, a poor prognostic sign and seemed, in our studies, to accompany progressive histologic derangement. Functional and histologic changes during the early phase of the disease, on the other hand, may be extremely poor without necessarily indicating a poor prognosis.

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The hemolytic effect of primaquine. XI. Decreased catalase activity in primaquine-sensitive erythrocytes

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Catalase activity was assayed in primaquine-sensitive and normal hemolysates. The results are at variance with those previously reported, due principally to refinements in technique. Bonnichsen, Chance, and Theorell, recognizing the extreme rapidity of the catalase reaction, emphasized that the reaction velocity between this enzyme and its substrate (H_2O_2) must be measured within seconds. The conditions necessary for attainment of a high degree of precision and reproducibility are described. The results were confirmed by a second method (sodium perborate as substrate) which does not require rapid measurement of reaction velocities.

Ten to 15 per cent of American Negro men have full expression of a genetically determined susceptibility to hemolysis by primaquine and other drugs; the catalase content of their erythrocytes was only 60 per cent of normal. Some, but not all, partially expressed women had decreased erythrocyte catalase activity. One atypical primaquine-sensitive Negro man had normal catalase activity, intermediate glucose-6-phosphate dehydrogenase activity, and intermediate susceptibility to hemolysis. The genetic significance of these findings and their relationship to the mechanism of drug-induced anemia is discussed.

Erythrocytes of certain individuals hemolyze in vivo following the administration of primaquine and therapeutic doses of many other compounds.[2, 3]

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Supported in major part by the Research and Development Command, Office of the Surgeon General, Department of the Army, under Contract DA-49-007-MD-566 with the Department of Medicine, University of Chicago, Alf. S. Alving, M.D., responsible investigator; and in part by Winthrop Laboratories, Inc., New York, N. Y.; and Eaton Laboratories, Inc., Norwich, N. Y.

A preliminary report of part of this work has been published.[1]

The investigations reported in this paper would not have been possible without the cooperation of the inmates and administrative staffs of the Illinois State Penitentiary, Stateville Branch, Joliet, and the State Reformatory for Women, Dwight, Ill.

Received for publication Oct. 12, 1960.

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Among those susceptible to hemolysis are 10 to 15 per cent of American Negroes and many other darkly pigmented people,[4] with a high incidence in tropical and semitropical countries; such susceptibility is rare in Northern Europeans. The predisposition to hemolysis is familial and is genetically transmitted as a sex-linked gene of intermediate dominance (variable expressivity).[5]

Because the initial clinical experiments which led to identification of this hemolytic syndrome were made by Hockwald and associates[6] during therapeutic experiments with the antimalarial drug, primaquine, this entity has been referred to as "primaquine-sensitive hemolytic anemia." The erythrocytes of individuals susceptible to hemolysis have been called "primaquine-sensitive" as well as "G-6-PD* deficient." The term "G-6-PD deficiency" is derived from the most characteristic abnormality of the erythrocytes in primaquine-sensitive† individuals.[7]

The drug-sensitive erythrocytes are characterized by several metabolic abnormalities, most of which center about the deficiency of G-6-PD in the oxidative pathway of carbohydrate metabolism. This subject has recently been reviewed by Carson.[8] Although knowledge of the mechanism of hemolysis is incomplete, the alterations in GSH metabolism and the decreased rate of regeneration of TPNH, both of which are related to the G-6-PD deficiency, have been considered to be intimately related to the vulnerability of the erythrocytes to hemolytic agents. All of these abnormalities are directly related to, or dependent upon, carbohydrate metabolism. We wish to report that the erythrocytes are also deficient in an enzyme which appears to be only distantly related, or entirely unrelated, to carbohydrate metabolism.

Materials and methods

All individuals participating in this investigation were Negro volunteers, either male inmates of the Illinois State Penitentiary, Stateville Branch, Joliet, or female inmates of the State Reformatory for Women, Dwight, Illinois. Primaquine-sensitivity or nonsensitivity had been ascertained in all cases by the daily administration of primaquine, 30 mg. base, for 14 days. None of the subjects had received hemolytic drugs for at least 4 months prior to the present studies.

Substrate A, hydrogen peroxide. An approximately 0.6 M solution was prepared by diluting 3.0 ml. Superoxol‡ up to 50.0 ml. with water. This substrate was prepared daily and the concentration determined by titration against $KMnO_4$.

Substrate B, sodium perborate. An approximately 0.1 M solution was prepared daily by dissolving 7.5 Gm. $NaBO_3 \cdot 4H_2O$* in 500 ml. aqueous solution. The pH was adjusted to 7.0 with concentrated hydrochloric acid (approximately 4 to 5 ml.).

Buffer. Phosphate buffer, .01 M, pH 7.0, was prepared by adding 61 ml. 0.1 M Na_2HPO_4 to 39.0 ml. 0.1 M KH_2PO_4 and bringing the total volume to 1,000 ml. with distilled water.

*Abbreviations: G-6-PD, glucose-6-phosphate dehydrogenase; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; and GSSG and GSH, oxidized and reduced glutathione, respectively.

†In this paper "primaquine-sensitive" refers to both men (hemizygous) and women (homozygous) who have full expression of the trait and who undergo an acute, severe in vivo hemolysis when a standard test course of primaquine, 30 mg. base, is given daily for 14 days. "Intermediate" refers to heterozygous women who undergo a less severe and more variable hemolysis than do individuals with full expression when a standard course of primaquine is administered.

‡Hydrogen peroxide solution 30 per cent, Merck Sharp & Dohme, Inc., Rahway, N. J.

The final pH was adjusted by adding one or the other stock phosphate solutions using a Beckman Model "G" pH meter.

Potassium permanganate. Standard solutions (approximately 0.1 N) were prepared from $KMnO_4$ crystals* as described by Kolthoff and Sandell.[9]

Preparation of hemolysate. Maximal enzyme activity was obtained by employing the preparative procedures outlined below. Venous blood, mixed with an anticoagulant,† was centrifuged at 3,000 g for 10 minutes at 4° C., and the plasma and buffy coat were discarded. The erythrocytes were washed 3 times with cold 0.145 M sodium chloride. After the final washing the packed erythrocytes were transferred to an Erlenmeyer flask and lysed by rapidly freezing (in an acetone-solid CO_2 bath) and thawing 3 times. After addition of 5 volumes of water the mixture was centrifuged at 20,000 g at 4° C. in order to pack the stromata. The upper half of the supernatant hemolysate was carefully siphoned off and saved for assay. This preparation was microscopically free of stromata. Hemoglobin determinations were performed in duplicate (alkaline oxyhemoglobin read at 545 mμ in a Coleman Junior spectrophotometer) and the hemolysate was diluted with water to a calculated level of 2.0 or 0.015 Gm. per cent, depending upon the method of assay to be used.

The above procedure was adopted after initial experiments revealed that catalase activity was 5 per cent greater when the hemolysate was prepared from washed, compared to unwashed, erythrocytes, and 2 to 5 per cent greater when lysis was produced by freezing and thawing compared to water (hypotonic) lysis. No loss of activity occurred if the hemolysate, with or without stromata, was assayed within a few hours of lysis. A 5 to 20 per cent loss of activity occurred if the hemolysate with stromata was heated to 37° to 43° C., compared to stromata-free hemolysates treated similarly. A 2 to 20 per cent loss of activity resulted from dialyzing stromata-free hemolysate 12 hours against 0.01 M phosphate buffer, pH 7.0. The effect of these procedures was approximately the same on sensitive and nonsensitive hemolysates.

Catalase assay A, hydrogen peroxide method. The titrimetric method introduced by Senter[10] in 1903, as employed by Von Euler[11] in 1934, was used with the following modifications. Three equal aliquots of hemolysate were incubated with the substrate (H_2O_2) in separate flasks under identical conditions; the reactions were stopped instantaneously at 5, 10, and 15 seconds, respectively, by the addition of acid through a syringe pipette. The disappearance of H_2O_2 was estimated oxidimetrically by titration against $KMnO_4$. Von Euler incubated substrate with enzyme in a single flask, removing aliquots for analysis at various times, a procedure which precludes accurate timing of short reaction intervals.

The assay was performed in a cold room at 4° C. Fifty milliliters of 0.01 M phosphate buffer, pH 7.0, and 2.0 ml. of approximately 0.6 M H_2O_2 were mixed in a 125 ml. Erlenmeyer flask (0.02 M final concentration H_2O_2). To ensure maximum accuracy, timing was controlled by a stopwatch operated by a second technician. At a signal, 0.5 ml. of the hemolysate preparation (2.0 Gm. per cent Hb.) was quickly (< 0.5 second) added from a blowout pipette during vigorous swirling of the mixture. The reaction was stopped at 5, 10, or 15 seconds (t′) by the rapid addition (< 0.2 second) of at least 1 ml. of 8 N H_2SO_4 from a syringe pipette. The remaining hydrogen peroxide was then titrated against 0.1 N $KMnO_4$. The H_2O_2 present initially (zero time, t^0) was measured in a mixture identical except that water was substituted for hemolysate or that hemolysate was added after H_2SO_4 acidification. Catalase activity, $C_{H_2O_2}$, was expressed as mEq. H_2O_2 decomposed by 0.5 ml. of 2.0 Gm. per cent hemolysate solution, and was computed as follows:

$$C_{H_2O_2} = N(X^0 - X')$$

where

N = normality of $KMnO_4$,

X^0 = ml. $KMnO_4$ needed to titrate H_2O_2 initially present (t^0),

*Fisher Certified, Fisher Scientific Company, New York, N. Y.

†Identical enzyme activity was obtained using either heparin or ACD as an anticoagulant or by defibrinating whole blood with glass beads.

and
X' = ml. $KMnO_4$ needed to titrate
H_2O_2 remaining after reaction (t').

Hemoglobin was chosen as a standard of reference for enzyme activity, in preference to unit number of cells or unit volume of cells, for the reasons given by Carson.[8]

CALCULATION OF MOLECULAR CONCENTRATION OF CATALASE IN ERYTHROCYTES. The concentration of catalase in a solution can be calculated by employing an equation given by Maehly and Chance:[12]

$$E = \frac{\ln (X^0/X)}{K'_1 \, t}$$

where E = concentration of enzyme in moles per liter, ln = natural logarithm, X^0 = substrate originally present,* X = substrate remaining after incubation with enzyme,* t = time in seconds, and $K'_1 = 2.8 \times 10^7$ (0° C.).†
The enzyme concentration (E) derived from this equation is in moles per liter of assay solution. The system of 52.5 ml. contained 0.5 ml. of a 2.0 Gm. per cent hemolysate as the enzyme source; the concentration of catalase per gram of hemoglobin or per erythrocyte can be easily computed.

Catalase assay B, sodium perborate method. The perborate method of Feinstein[14] was used as a second method of catalase assay. Five milliliters of approximately 0.1 M $NaBO_3 \cdot 4H_2O$ (Fisher Reagent), made fresh daily‡ and adjusted to pH 7.0, and 4.0 ml. of 0.1 M phosphate buffer, pH 7.0, were mixed in a 50 ml. Erlenmeyer flask and placed in a water bath at 37° C. Five minutes were allowed for temperature equilibration, then 1.0 ml. of the hemolysate-enzyme preparation (0.015 Gm. per cent Hb.) was quickly (< 0.5 second) added from a blowout pipette. At the end of the incubation period, t' (1, 2, or 5 minutes), the reaction was stopped by rapid (< 0.2 second) addition of 2 ml. of approximately 8 N H_2SO_4, delivered from a syringe pipette. The remaining perborate was then measured by titration against 0.1 N $KMnO_4$. The quantity of $NaBO_3 \cdot 4H_2O$ initially present (zero time, t^0) was measured identically except that water was substituted for hemolysate or hemolysate was added after H_2SO_4 acidification. Catalase activity ($C_{NaBO_3} \cdot 4H_2O$) was expressed as mEq. $NaBO_3$ decomposed as follows:

$$C_{NaBO_3} \cdot 4H_2O = N(X^0 - X')$$

where
N = normality of $KMnO_4$,
X^0 = ml. $KMnO_4$ needed to titrate
$NaBO_3$ initially present (t^0),
and
X' = ml. $KMnO_4$ needed to titrate
$NaBO_3$ remaining after reaction (t').

The figures given in Table IV represent the results of 1 minute reactions.

Glucose-6-phosphate dehydrogenase activity. G-6-PD was assayed spectrophotometrically by a modification of the method of Kornberg and Horecker.[15] The assay mixture of 3.05 ml. contained hemolysate (1 mg. hemoglobin, stroma not removed) as the enzyme source, 2 μM glucose-6-phosphate,§ 0.4 μM TPN,§ 30 μM $MgCl_2$, and 200 μM Tris buffer,§ pH 8.0. The rate of TPN^+ reduction to TPNH at 24 ± 2° C. was determined by recording the increased

*The milliliter of $KMnO_4$ used in the titration of H_2O_2 can be substituted directly for X^0 and X, respectively.

†Maehly and Chance,[12] using Herbert and Pinsent's[13] data, have determined that K'_1 for crystalline human erythrocyte catalase is 3.4×10^7 (20° C.); since our assays were performed near 0° C., and $Q_{10} = 1.1$ for catalase, K'_1 in our system becomes 2.8×10^7 (0° C.).

‡Sodium perborate, 0.1 M, used fresh or stored for 1 week at 4° C., gave identical results.

§Sigma Chemical Company, St. Louis, Mo.

absorption at 340 mμ in a Beckman DU spectrophotometer (Silica cuvettes, 1.0 cm. light path) in 60 minutes with a photomultiplier attachment. The values given in Table IV represent the change in optical density under these conditions.

Glutathione stability test. The method devised by Beutler,[16] with strict adherence to the precautions outlined by Flanagan and associates,[17] was employed. The results are expressed as milligrams GSH per 100 ml. R.B.C. remaining after incubation for 2 hours with acetylphenylhydrazine.

Table I. Catalase assays of hemolysates by the hydrogen peroxide titrimetric method

Amount 0.0997N $KMnO_4$ required to titrate substrate (H_2O_2) remaining (ml.)				
		After reaction with hemolysate		
Case No.	*Initially*	*5 seconds*	*10 seconds*	*15 seconds*
Normal men				
1	23.56	14.70	10.59	6.20
2	23.56	15.13	10.93	6.99
3	23.25	15.20	10.95	6.79
4	23.25	14.31	10.51	6.01
5	23.19	14.10	10.33	6.49
6	23.61	14.28	10.49	6.83
7	23.36	14.35	10.41	6.14
8	23.61	15.04	10.00	6.41
9	23.41	14.43	10.32	6.28
Mean	23.34	14.62	10.50	6.46
S. D.	± .18	± .39	± .28	± .32
Primaquine-sensitive men				
1	23.19	16.76	13.00	8.76
2	23.20	16.31	12.82	8.40
3	23.20	16.52	13.55	9.21
4	23.40	17.62	14.01	9.71
5	23.40	16.66	12.96	8.88
6	23.55	16.27	12.40	8.40
7	23.55	17.10	13.00	9.93
8	23.35	16.89	13.50	10.21
9	23.45	16.64	13.72	10.24
Mean	23.37	16.75	13.22	9.32
S. D.	± .14	± .27	± .48	± .67

Table II. Catalase concentration measured at different reaction times

	Enzyme concentration (moles per liter of reaction mixture)	
Reaction time (seconds)	*Normal men*	*Primaquine-sensitive men*
5	3.3×10^{-9}	2.3×10^{-9}
10	2.9×10^{-9}	2.0×10^{-9}
15	3.1×10^{-9}	2.2×10^{-9}
30	2.8×10^{-9}	1.9×10^{-9}
60	2.1×10^{-9}	1.6×10^{-9}

These calculations were made using the formula given in *Materials and Methods*, section entitled "Calculation of molecular concentration of catalase in erythrocytes."

Chromium^{51} erythrocyte survival rate. Fifty milliliters of whole blood (coagulation prevented with heparin or acid-citrate–dextrose) was incubated aseptically with 120 μc Cr^{51} (Rachromate*) at room temperature for 1 hour. The labeled blood was then administered intravenously to the donor. Approximately 12 to 24 hours after the infusion, and subsequently, at 48 hour intervals, duplicate 3 ml. samples of whole blood were obtained for determination of the residual Cr^{51} radioactivity. The samples were lysed by freezing and thawing and were stored at 5° C. The counts per minute were determined at weekly intervals in a Nancy Wood scintillation detector and were corrected for isotope decay (but not for elution) by comparison with a standard Cr^{51} solution.

Critique of methods

Senter,[10] in 1903, and Von Euler,[11] in 1934, allowed the incubation of enzyme with substrate to proceed for 5 minutes or longer. In 1947, Bonnichsen, Chance, and Theorell[18] appreciating the extraordinary speed of the catalase reaction, called attention to the necessity of measuring the reaction rates after only seconds of incubation in order to avoid substrate depletion. They also pointed out that considerable inactivation of enzyme by substrate occurs during incubation and that a relatively high concentration ratio of enzyme to substrate minimizes the relative importance of this inactivation. These points were also stressed in a definitive review by Maehly and Chance, in 1954.[12]

More than 50 per cent of the H_2O_2 was decomposed by catalase in the hemolysate preparation within the first 15 seconds of the reaction. In fact, accurate enzyme assay was precluded, under the conditions of this assay, when incubation periods greater than 30 seconds were used. However, it was possible to measure reaction rates with macrotechniques at 5 second intervals by using separate flasks for each incubation and stopping the reaction by the rapid addition of sulfuric acid through a syringe pipette.

The perborate method of Feinstein,[14] which correlates well with the H_2O_2 method, was chosen as a second method of catalase assay. Ephraim[19] has postulated that the formula for perborate may be written $NaBO_3 \cdot 3H_2O \cdot H_2O_2$; probably a small amount of H_2O_2 is continually generated from the perborate as the catalase reaction proceeds. The inactivation of catalase by $NaBO_3 \cdot 4H_2O$ is relatively minor,[14] probably because the concentration of H_2O_2 in the assay mixture never reaches critical levels. The assay need not be performed at cold temperatures and longer reaction times may be used than with the hydrogen peroxide method.

The reproducibility in duplicate samples by either the hydrogen peroxide or sodium perborate methods is ± 1 per cent. The daily variation was practically nil; catalase assays were performed twice weekly on 4 men for 3 months, and the highest and lowest values fell within ± 3 per cent of the mean for the entire period for each man. Reproducibility of this order was obtained in approximately 12,000 assays during 1958.†

Results

Catalase activity and content in hemolysates from adult Negro men: hydrogen peroxide method.

A. ACTIVITY. The activity of catalase in hemolysates of primaquine-sensitive

*Rachromate, Abbott Laboratories, North Chicago, Ill.

†In the latter part of February, 1959, fresh hemolysate preparations suddenly and without apparent cause gave only half the catalase activity that had been observed in the previous 6 months. Enzyme concentration studies and kinetic curves became uninterpretable. The catalase in hemolysates, which had previously remained stable when stored overnight at 4° C., now lost half its activity in a few hours. Sensitivity was so grossly impaired and results so erratic that differences of activity of the order of 20 to 40 per cent formeraly obtained fell within the analytical error of the method. During the next several months the catalase activity of our hemolysate preparations slowly increased but has not returned to previous levels, accuracy, or reproducibility. During the period when the assay was least sensitive and the results most erratic the differences in activity between normal and primaquine-sensitive hemolysates was usually demonstrable, but to a variable degree. Many inhibitors of catalase are known,[20] i.e., anions, cyanide, sodium hydrosulfide, azide, hydroxylamine, ascorbic acid, sulfhydryl compounds, BAL (dimercaprol), and others. Some of these compounds are used regularly in our laboratory (cyanide and GSH daily, azide, ascorbic acid, and BAL intermittently), and possibly trace amounts of an inhibitor may be interfering with the assay.

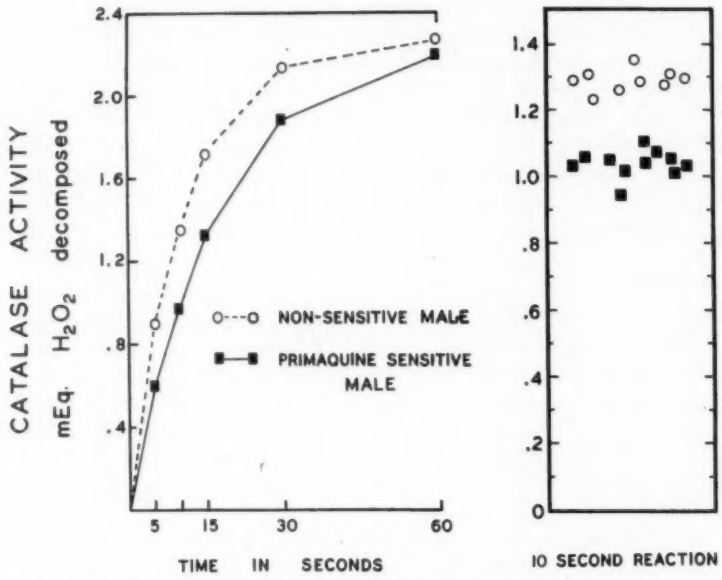

Fig. 1. A, A "time" study of the decomposition of hydrogen peroxide by hemolysate catalase. Approximately half of the initial concentration (2.3 mEq.) of H_2O_2 was consumed by normal hemolysates within 10 seconds. *B*, Catalase activity of hemolysates from 9 normal and 11 primaquine-sensitive Negro men.

men (hemizygous) was less than that in nonsensitive men, as measured in kinetic studies (Fig. 1, *A*). The substrate consumed by hemolysates from each of 11 primaquine-sensitive men was 80 per cent of that consumed by each of 9 nonsensitive men (Fig. 1, *B*) measured at the 10 second reaction point under the conditions of assay described. This difference prevailed over a broad range of pH (5.8 to 8.4) and hemolysate concentration (0.5 to 8.0 Gm. per cent Hb.).

B. CONTENT. The catalase content in men has been calculated (Tables II and III) for normal and primaquine-sensitive erythrocytes using the raw assay data in Table I (for mathematical treatment, see *Materials and Methods*). Our finding, that catalase comprises 0.34 per cent of the dry weight of normal, fresh human erythrocytes, is in approximate agreement with the figure of 0.26 per cent reported by Herbert and Pinsent.[13] Their method of assay was not given. Mean catalase content of primaquine-sensitive erythrocytes is approximately 70 per cent of normal.

Catalase activity in hemolysates from adult Negro men: sodium perborate method. Decreased catalase activity in primaquine-sensitive hemolysates was also demonstrated, using $NaBO_3 \cdot 4H_2O$ as a source of substrate (Fig. 2, *A*). The average substrate consumed by hemolysates from each of 17 men (Fig. 2, *B*) was 60 per cent of that by each of 34 nonsensitive men as measured at the 1 minute reaction point. This difference in activity was apparent over a wide range of pH (5.8 to 8.4) and hemolysate concentration (0.005 to 0.1 Gm. per cent Hb.). However, one primaquine-sensitive male patient (H. S.) had normal erythrocyte catalase activity. He will be discussed below in the section "Partially expressed primaquine-sensitivity in a Negro man."

Table III. Catalase content of erythrocytes

Catalase	*Normal men*	*Primaquine-sensitive men*
Moles per R.B.C.*	4.8×10^{-19}	3.4×10^{-19}
Grams per R.B.C.*	10.6×10^{-14}	7.5×10^{-14}
% Dry weight R.B.C.*	0.34%	0.24%
% Of nonhemoglobin protein*	13.6%	9.6%

*In a single normal human erythrocyte[32] the hemoglobin content has been estimated to be 29 $\mu\mu g^2$; the dry weight, 30.885×10^{-12} Gm.[32]; and the nonhemoglobin protein, 7.8×10^{-12} Gm.[2] The molecular weight of normal human erythrocyte catalase is 220,000. We have assumed these figures to be the same in primaquine-sensitive as in normal erythrocytes.

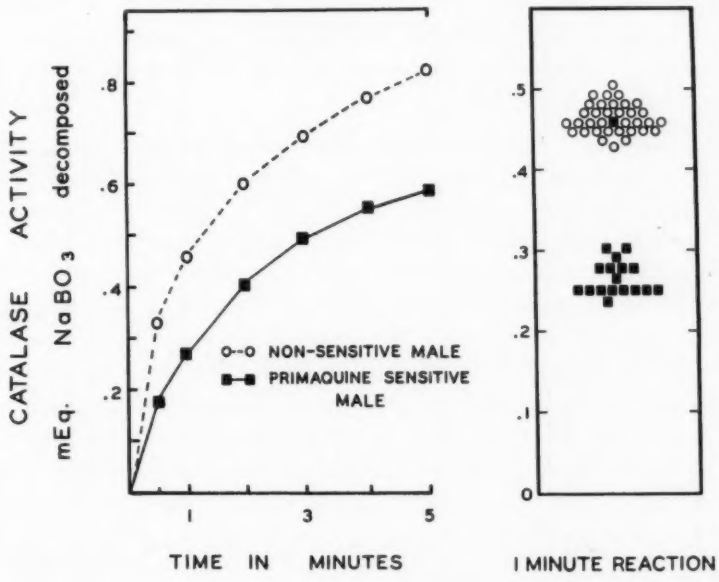

Fig. 2. A, A "time" study of the decomposition of sodium perborate by hemolysate catalase. Approximately half of the initial concentration (0.95 mEq.) of $NaBO_3 \cdot 4H_2O$ was consumed by normal hemolysate in one minute. *B,* Hemolysate catalase activity from 34 normal, 1 intermediate (atypical), and 17 primaquine-sensitive Negro men.

Catalase activity in hemolysates from adult Negro women. Eighty-three women were screened for primaquine-sensitivity. Of these, only one fully expressed (homozygous) primaquine-sensitive woman was found. No sharp division could be made between normal and intermediate (heterozygous) women. In some instances it was not possible to determine whether a woman was normal or intermediate either by any of the known laboratory tests or on the basis of clinical hemolysis with primaquine. It was possible, however, to determine with certainty that 8 women were intermediate* (Table IV). All of the intermediate women had hemolysate G-6-PD activity at least 2 standard deviations below the mean of normal men and at least 3 standard deviations above the mean of primaquine-sensitive men; some of them, however, had normal GSH stability tests. The 8 women had a more moderate hemolysis than primaquine-

*Probably a low estimate of the occurrence of heterozygosity because of the uncertainty of diagnosing mild intermediates.

Table IV. Variability of erythrocytic abnormalities and of drug-induced hemolysis among several different phenotypes of primaquine-sensitivity

Subjects		*Catalase activity*	*G-6-PD activity*	*GSH stability test*	*Hemolysis 30 mg. Primaquine daily for 14 days*		
					Reticulocytosis (peak value)	*Hematocrit (fall mm.)*	*R.B.C. survival (Cr^{51} half-life)*
Men							
Normal	mean	.46	.701	54	2.4	4.7	25
	S. D.	.01	.136	7	.2	2.0	2
	range	.43—.50 (34)*	.487—1.005 (20)	43—74 (20)	1.6—3.0 (6)	1.4—6.7 (4)	22—29 (6)
Intermediate H. S.† (1)	mean	.47	.210	28	6.2	9.0	16
	range	.46—.47	.193—.221	24—36			
Sensitive	mean	.27	.075	9	10.6	15.0	11
	S. D.	.01	.011	1	2.1	1.8	2.5
	range	.25—.30 (17)	.058—.094 (5)	7—10 (6)	6.8—13.4 (8)	11.5—17.3 (7)	9—17 (7)
Women							
Intermediate Normal catalase (6)	mean	.46	.380	38	5.2	5.9	18.5
	S. D.	.01	.040	7	1.2	2.3	1.5
	range	.46—.48	.308—.424	30—49	3.6—7.0	3.0—9.8	16—21
Intermediate Decreased catalase	M. B.†	.27	.422	37	6.0	9.0	17
	A. L.†	.27	.380	26	6.2	5.0	17
Sensitive (1)	R. P.†	.26	.041	10	22.9	16.5	11

*The number of individual cases is indicated in parentheses.
†Biochemical values for single cases are the averages of at least 3 determinations.

sensitive men when 30 mg. primaquine was administered daily, but the severity of hemolysis varied widely and the correlation of severity with G-6-PD activity was poor.

Three of the 83 women had decreased catalase activity; 2 of these were heterozygous (intermediate) and one was homozygous (Table IV). The heterozygotes having low catalase activity (Patients M. B. and A. L.) demonstrated a slightly greater degree of hemolysis than all but one of the intermediate women, although most of the others had a more severe deficiency of G-6-PD.

The third woman (Patient R. P.) who had decreased catalase activity, a markedly deficient G-6-PD activity, and a markedly positive GSH stability test was classified as homozygous. Her G-6-PD deficiency was more severe than that of the hemizygous men and she underwent primaquine-induced hemolysis which was at least as severe, and possibly more severe, as the hemolysis in sensitive men.

Partially expressed primaquine-sensitivity in a Negro man. One case (Patient H. S.) was of unusual interest. Of 18 affected men studied only he had normal catalase activity. In addition, the other abnormalities in his erythrocytes were less severe than in 75 additional primaquine-sensitive men who have been studied in this laboratory, but the G-6-PD deficiency of his erythrocytes was more severe than found in intermediate (heterozygous) women (Table IV). The degree of hemolysis experienced by this Negro man was less marked than by other sensitive men (Table IV); his hemolysis was of the same degree as the most marked hemolysis seen in intermediate women. The father of this volunteer is nonsensitive but his mother is primaquine-sensitive (presumably homozygous). Therefore, Patient H. S. is hemizygous, although his expression is atypical. Studies of other members of this family are in progress.

Discussion

Beutler and colleagues[21] reported that the activity of catalase in hemolysates from primaquine-sensitive Negroes was normal; Szeinberg and associates[22] obtained similar results in affected Caucasians. The disagreement between their results and ours, which show a 40 per cent decrease in catalase content, can probably be explained by differences in technique, especially our use of short incubation periods and other precautions which Bonnichsen, Chance, and Theorell[18] determined were necessary for valid assay.

In screening 200 American Negroes, decreased catalase activity was present only in association with primaquine-sensitivity, either completely or partially expressed. On the other hand, some intermediate women have normal catalase activity.

According to the one gene–one enzyme hypothesis, a mutation exerts a primary effect on the polypeptide structure of only one protein (enzyme). Of several possible explanations for the dual enzyme deficiency in primaquine-sensitive erythrocytes the most likely is that of pleiotropia, i.e., the activity, or synthesis, of both catalase and G-6-PD may be affected in common by a single mutant genetic determinant.

The relationship of acatalasemia, characterized by the complete absence of erythrocyte catalase activity in the fully expressed state,[23] to primaquine-sensitivity, characterized by decreased, but not absent, catalase activity, is speculative. Both disorders are transmitted by partially dominant genes, but their loci are different; that of acatalasemia being on an autosomal chromosome, that of primaquine-sensitivity on a sex (X) chromosome. The molecular diseases, therefore, may illustrate the participation of more than one gene in the production of a single enzyme. The possibility that the decreased catalase activity is merely a secondary manifestation of the primary enzymatic defect, that of G-6-PD, cannot be excluded.

The genotype of the atypical man, Patient H. S., is obscure. The normal catalase activity in his erythrocytes may modify the phenotype. This hypothesis is supported by our observations that, with a single exception, intermediate women having normal catalase activity generally experienced a less severe hemolysis than did those having decreased catalase activity.

Our studies also suggest that the presence of 2 mutant alleles in homozygous women, as illustrated by Patient R. P., results in a more severe deficiency of G-6-PD and a greater susceptibility to drug-induced hemolysis than does the single mutant allele in hemizygous men. More cases are needed to test this hypothesis.

The production of H_2O_2 in biological systems is brought about by the transfer of hydrogen to oxygen by several oxidative enzyme systems, i.e., the oxidation of glucose by glucose oxidase, and by the auto-oxidation of flavoproteins, sulfhydryl compounds (GSH), or ascorbic acid.[24] Hydrogen peroxide may also be formed enzymatically in erythrocytes by the reaction between oxygen and TPNH.[25]

The concentration of catalase in human erythrocytes (13 per cent of the nonhemoglobin protein) is higher than in any other tissue, suggesting that catalase has an important function in red cell metabolism. Until recently it had been believed that the sole function of catalase was to decompose hydrogen peroxide to molecular oxygen and water. However, catalase may be involved in carbohydrate metabolism by acting as a peroxidase in several oxidation-reduction reactions. Keilen and Hartree[26] reported that hydrogen peroxide, in the presence of catalase, can effect the oxidation of alcohols with the formation of formaldehyde and water. Also, Francouer and Denstedt[27] demonstrated that ribose-5-phosphate of erythrocytes can be oxidized by a similar mechanism.

Jandl[28] has recently presented evidence which indicates that the initial step toward death of red cells is the oxidation of hemoglobin to methemoglobin. Catalase can protect hemoglobin from oxidation to methemoglobin, as shown by Foulkes and Lemberg[29] and Takahara.[30] Excessive hydrogen peroxide, allowed to accumulate because of decreased catalase activity, could contribute to the oxidation of hemoglobin and other cellular components. Brewer[31] has recently reported that primaquine-sensitive erythrocytes accumulate more methemoglobin in vivo in response to sodium nitrite administration than do normal cells. The combination of excessive methemoglobin formation due to the deficiency of

catalase, with the diminished rate of methemoglobin reduction due to the diminished rate of TPNH regeneration, may enhance the vulnerability of G-6-PD deficient cells to destruction. The prime importance of deficient activity of G-6-PD in primaquine-sensitive erythrocytes is emphasized by the pivotal role that hemoglobin-methemoglobin metabolism appears to have in drug-induced hemolysis. Further studies are needed to define the significance of catalase deficiency in the complex of abnormalities in primaquine-sensitive erythrocytes.

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The hemolytic effect of primaquine. XII. Shortened erythrocyte life span in primaquine-sensitive male Negroes in the absence of drug administration

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Older erythrocytes of primaquine-sensitive Negro men are susceptible to in vivo hemolysis by many drugs. The enzyme glucose-6-phosphate dehydrogenase is deficient in these cells; they also differ from normal cells in many other respects. Earlier investigators believed that the abnormalities in these erythrocytes were harmless unless drugs were administered. The present study, utilizing DFP^{32} and Cr^{51} labeling techniques, demonstrates that these cells die prematurely in vivo even when not stressed by the presence of drugs. The finding of a shortened survival of primaquine-sensitive erythrocytes increases the similarity between this condition and certain cases of congenital nonspherocytic hemolytic anemia. These studies indicate that glucose-6-phosphate dehydrogenase, or the pentose phosphate pathway of which it is a part, plays an important role in maintaining the red cell through a normal life span.

Primaquine sensitivity (also called erythrocyte glucose-6-phosphate dehydrogenase deficiency) is an inherited abnormality of metabolism which occurs in a varying percentage of the members of all races. These individuals experience intravascular hemolysis when they are administered certain drugs, including primaquine.[1-3] Family studies show that inheritance of the primaquine-sensitive trait is by a sex-linked gene of intermediate dominance.[4, 5]

Erythrocytes of sensitive individuals have several biochemical abnormalities.

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Supported in major part by the Research and Development Command, Office of the Surgeon General, Department of the Army, under Contract DA-49-007-MD-566 with the Department of Medicine, University of Chicago, Alf S. Alving, M.D., responsible investigator; and in part by Winthrop Laboratories, New York, N. Y.; Parke, Davis & Company, Detroit, Mich.; and Eaton Laboratories, Inc., Norwich, N. Y.

The investigations reported in this paper would not have been possible without the cooperation of the inmates and administrative staff of the Illinois State Penitentiary, Stateville Branch, Joliet, Ill.

Received for publication Oct. 17, 1960.

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These abnormalities are uniformly present in the red cells of almost all affected Negro men (presumably hemizygous); however, in heterozygous Negro women there is considerable variation between individuals, and, in some, the abnormalities are undetectable.[5] A decrease in hemolysate glucose-6-phosphate dehydrogenase (G-6-PD) activity is the most characteristic abnormality discovered to date,[6] but it also is within normal limits in some heterozygous Negro women.[5, 7] This paper is a report of observations on the natural life span of erythrocytes of normal and hemizygous primaquine-sensitive Negro men who previously have been shown to manifest major expression of the genetic defect.

In early studies the biochemical abnormalities appeared to be harmless unless a drug was administered.[8] Analysis of reticulocyte counts, hematocrit levels, and chromium^{51} (Cr^{51}) survival curves revealed no evidence of increased hemolysis, or shortening of the red cell life span, in sensitive Negro men.[2, 3, 9, 10] In reviewing Cr^{51} survival studies collected in this laboratory as part of later programs, the data suggested that primaquine-sensitive red cells had a shorter than normal survival. Therefore, studies were initiated utilizing both the Cr^{51} and diisopropylfluorophosphate^{32} (DFP^{32})* technique of labeling red cells to establish more accurately the life span of primaquine-sensitive erythrocytes.

Materials and methods

Volunteer subjects. Healthy primaquine-sensitive or nonsensitive Negro volunteers at the Illinois State Penitentiary participated in this investigation. All sensitive individuals had major expression of the trait and were, therefore, hemizygous. At least 11 months had elapsed since the volunteers had received hemolytic drugs. Withdrawal of blood was limited to the approximately 15 ml. per week which was required for radioisotope counting. Normal control subjects were studied concurrently.

DFP^{32} labeling technique. DFP^{32}, with a specific activity of 119 μc per milligram of DFP, was obtained as a dilute solution of approximately 1 mg. of DFP^{32} per milliliter of sterile anhydrous propylene glycol. Our technique of intravenous administration for in vivo labeling was the same as that of Athens and associates[11]; the amount administered was approximately 200 μc DFP^{32} per volunteer.

DFP^{32} counting technique. The day (day zero) after the administration of DFP^{32}, and every subsequent fourth day, 8 ml. of blood was taken from the antecubital vein of each subject. Heparin was used as an anticoagulant. Six milliliters of the blood was pipetted into test tubes which were accurately calibrated with a scratch mark at the 5 ml. level. The blood was centrifuged at 4° C. in this test tube at 1,000 g in an International PR-2 centrifuge for 15 minutes, and the plasma was removed without disturbance of the buffy coat. Ten milliliters of 0.85 per cent sodium chloride solution was added and the cells were resuspended and centrifuged again in the cold at 575 g for 15 minutes; the supernatant was then removed without disturbing the buffy coat. This procedure was repeated twice, making a total of 3 washings. It is advisable to wash the cells before counting, in order to eliminate any labeled plasma proteins as well as to decrease their internal absorption (p^{32} is a β-emitting isotope). After the third removal of the supernatant, distilled water was added to exactly the 5 ml. calibration mark, and the cells were resuspended and placed in a freezer at –20° C. overnight. The next day the sample was warmed to room temperature and then mixed by repeated inversion. Two milliliter aliquots of the sample were accurately pipetted into each of duplicate counting planchets† (copper sample pans 1¼ inches in diameter by $\frac{3}{32}$ inch deep, with raised concentric annules on the floor to prevent welling of the sample during the drying procedure). The samples in these duplicate planchets were dried slowly (to prevent spat-

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Table I. Statistical analysis of the erythrocyte survival time as measured by the Cr^{51} technique (no drugs administered)

Subjects	*Half life (days)*	*Range*	*Mean*
Normal			
Pow.	29.5	31.0 – 25.5	28.7
Ban.	29.0		
Del.	25.5		
Wil.	31.0		
Primaquine-sensitive			
Dew.	18.0	24.5 – 18.0	20.2
Jac.	24.5		
Ree.	18.0		

$t = 3.8$; p is between 0.02 and 0.01.

tering) for a period of 2 hours at a distance of 22 inches under a 250 watt infrared lamp. The planchets were then placed in an oven at 98° C. and dried for an additional 2 hours. After cooling, they were counted for 1 minute in an end window gas flow counter having an efficiency of approximately 30 per cent of the total disintegrations (60 per cent of those directed upward) and a background of 20 c.p.m.; counts of the duplicate samples were averaged. Correction for radioisotope decay was made by preparing a standard planchet of inorganic orthophosphate p^{32} (with approximately the same per minute count on day zero) and counting it every day on which counts were made thereafter. After correcting the counts for background, the percentage of cells surviving on any given day (day X) was then calculated according to the following formula:

$$\text{per cent of cells surviving} = \frac{\text{c.p.m. of standard on day zero}}{\text{c.p.m. of standard on day X}} \times \frac{\text{c.p.m. of sample on day X}}{\text{c.p.m. of sample on day zero}} \times 100.$$

Cr^{51} labeling and counting technique. Our method of labeling with and counting Cr^{51} has been previously described.[12]

Results

Cr^{51} red cell survival. The 50 per cent survival times (erythrocyte half lives), as determined by the Cr^{51} method of 4 normal and 3 primaquine-sensitive individuals, are compared in Table I. The mean of the erythrocyte half lives in primaquine-sensitive individuals is significantly shorter (at the 2 per cent level of confidence) than the normal mean. These 7 cases were studied simultaneously. Although there is no overlap of the 2 groups, a larger number of cases studied over a period of several years has shown considerable overlap; the means, however, remain significantly different.

DFP^{32} red cell survival. Analysis of the erythrocyte half lives of 5 normal and 4 primaquine-sensitive Negro men,* as determined by the DFP^{32} technique, demonstrated a highly significant shortening (at the 1 per cent level of confidence) of the life span of primaquine-sensitive erythrocytes (Table II). The erythrocyte half lives of the normal individuals range from 77 to 59 days, with

*These are not the same individuals discussed in the Cr^{51} section.

Table II. Statistical analysis of the erythrocyte survival time as measured by the DFP^{32} technique (no drugs administered)

Subjects	*Half life (days)*	*Range*	*Mean*
Normal			
Com.	65.5		
Dav.	77.0		
Wig.	64.0	77 – 59	66.1
Sim.	59.0		
Wil.	65.0		
Primaquine-sensitive			
Jac.	46.0		
Wea.	57.5	58 – 38	48.0
Rad.	38.0		
Hun.	50.5		

$t = 3.6$; p is between 0.01 and 0.001.

a mean of 66 days; the half lives of the primaquine-sensitive erythrocytes range from 38 to 58 days, with a mean of 48.2 days. Fig. 1 shows the average and range of the 2 groups of DFP^{32} curves.

Discussion

Critique of methods. Cr^{51} possesses an inherent disadvantage as an erythrocyte label; it elutes from the cells at a rate of about 1 per cent per day,[13] thus shortening the apparent erythrocyte half life of normal individuals from an expected 60 to approximately 30 days. In addition, a graph of the Cr^{51} content of the red cells as a function of time in normal individuals is bowed rather than linear, and frequently shows a large early decrease, thus making difficult any comparison of one curve with another. This rapid early loss of Cr^{51} from apparently intact cells is variable.[14] Therefore, although Cr^{51} is a very useful label for studies involving marked changes of erythrocyte survival, elution and variability in the shape of the curve make difficult the detection and interpretation of small abnormalities of red cell survival.

DFP^{32} was first used as a red cell label by Cohen and Warringa,[15] who

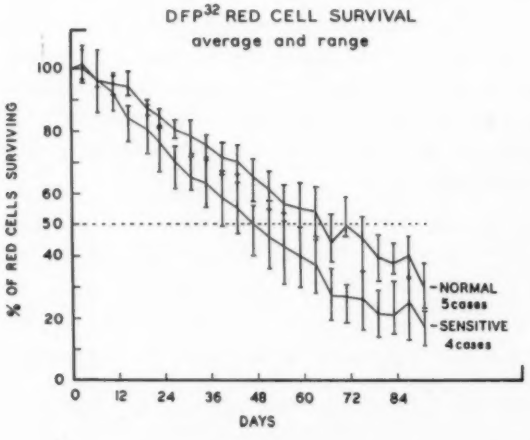

Fig. 1. Red cell survival in 5 normal and 4 sensitive subjects as determined by the DFP^{32} technique. The vertical lines represent the range for each group on that day; the sloping lines are drawn through the mean value for each day (no drugs administered).

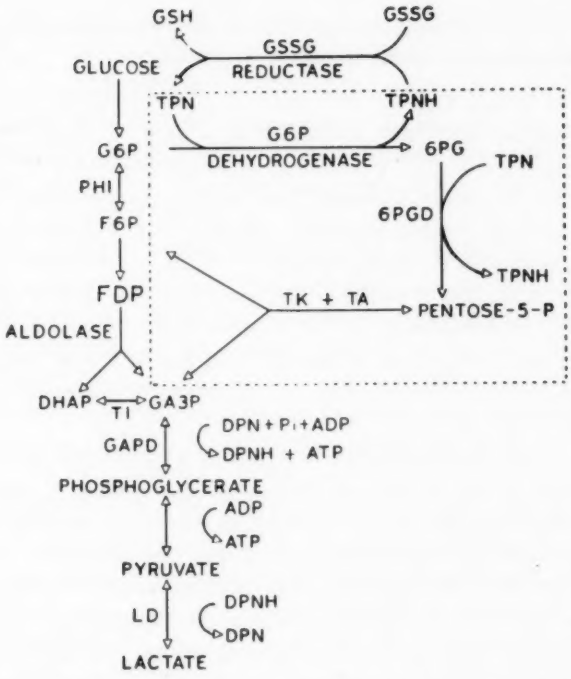

Fig. 2. Pathways of carbohydrate metabolism in the mature human erythrocyte. The vertical series of reactions at the left represent the Embden-Meyerhoff glycolytic pathway. The reactions enclosed within the dotted line represent the pentose phosphate pathway. Abbreviations: *GSH, GSSG,* reduced and oxidized glutathione, respectively; *TPNH, TPN,* reduced and oxidized triphosphopyridine nucleotide, respectively; *DPNH, DPN,* reduced and oxidized diphosphopyridine nucleotide, respectively; *G6P,* glucose-6-phosphate; *6PG,* 6-phosphogluconate; *6PGD,* 6-phosphogluconic dehydrogenase; *pentose-5-P,* pentose-5-phosphate; *TK,* transketolase; *TA,* transaldolase; *FDP,* fructose diphosphate; *PHI,* phosphohexose isomerase; *F6P,* fructose-6-phosphate; *DHAP,* dihydroxyacetone phosphate; *GA3P,* glyceraldehyde-3-phosphate; *TI,* triose isomerase; *GAPD,* glyceraldehyde phosphate dehydrogenase; *Pi,* inorganic phosphate; *ADP, ADT,* adenosine diphosphate and triphosphate, respectively; *LD,* lactic dehydrogenase.

showed that a fraction of the DFP^{32} becomes irreversibly bound to the red cells and that this fraction disappears from the blood at a rate corresponding to the normal replacement of the red cells. Utilizing this technique, red cell life spans of 116 and 129 days were found in 2 normal individuals. Bove and Ebaugh[16] obtained similar results (123.6 ± 11.0 days) in 8 normal subjects. DFP^{32} possesses characteristics which make it a good red cell label. It binds to certain protein enzymes of the cell, such as esterases, and when the cell is destroyed, the label is excreted as diisopropylphosphate^{32} and is not reutilized.[15] The finding of a normal cholinesterase activity in sensitive erythrocytes by Tarlov and associates[17] lends validity to the use of DFP^{32} in primaquine sensitivity, since some of the DFP^{32} binds to this enzyme. Hjort and co-workers[18] and others[16, 19] have found that there is some elution of DFP^{32} from the erythrocyte, but this is dependent upon the dose used and, in any case, is limited to the first few days after administration and does not interfere with long term studies of cell survival.[14, 18] The lack of chronic elution is the principal reason

that sufficient radioactivity remains on the red cells to make accurate counts for as long as 90 days after the administration of DFP^{32}.

Significance of results. Energy for the mature human red cell is derived from 2 pathways of carbohydrate metabolism (Fig. 2). Under physiologic conditions, approximately 90 per cent of glucose appears to be utilized by the anaerobic glycolytic Embden-Myerhoff pathway, and the other 10 per cent, by the oxidative pentose phosphate pathway (PPP).[20] Until recently, the PPP has been considered insignificant in the mature erythrocyte under physiologic conditions,[21] but Murphy[20] has recently estimated that the pathway's contribution to potential energy is significant. The shortened survival of the sensitive red cells demonstrates the importance of the PPP in maintaining the integrity of the erythrocyte, since most of the biochemical abnormalities in these cells are concerned with this pathway.

The PPP is the only source for generation of reduced triphosphopyridine nucleotide (TPNH) in the mature erythrocyte; this occurs at 2 steps, but if the first, G-6-PD, is lacking, the next, 6-phosphogluconic dehydrogenase, is ineffective because it has no substrate (Fig. 2). Thus, the deficiency in G-6-PD leads to an inability to reduce triphosphopyridine nucleotide to TPNH in the sensitive cell. The known functions of TPNH include participation in the reduction of methemoglobin, the reduction of glutathione, the degradation of drugs, and lipid synthesis (this latter occurs mostly in reticulocytes and immature nucleated erythrocytes,[22] but also, to a slight extent, in mature erythrocytes[23]). The failure to generate TPNH is probably a major factor in the sensitive cell's premature death and susceptibility to hemolysis by drugs.

The decreased survival of G-6-PD deficient erythrocytes may represent an acceleration of the normal mechanism for red cell destruction. The aging or length of the life span of cells may be determined by the maintenance of activity of one or more enzymes, as has been postulated by several investigators.[24-26] Marks and Gross[27, 28] have shown that the activity of G-6-PD declines with age in the erythrocytes of both normal and primaquine-sensitive individuals. The activities of glyoxalase,[24] catalase,[24] cholinesterase,[24, 29] aldolase,[26] phosphohexose isomerase,[25, 26] and 6-phosphogluconic dehydrogenase[25] also have been reported to be lower in old erythrocytes than in young cells of normal individuals.

The shortened erythrocyte life span in primaquine-sensitive individuals indicates that the mean cell age of the circulating erythrocytes is less in them than in normal persons. It is, therefore, necessary to evaluate small biochemical or other differences in these cells in the light of this observation.*

The primaquine-sensitive male Negro has chronic hemolysis which differs in degree only from that seen in certain cases of congenital nonspherocytic hemolytic anemia (CNSHA) with G-6-PD deficiency of a greater magnitude.[31, 32] The primaquine-sensitive man is not anemic because the mild hemolysis is masked by a compensatory hematopoiesis.

*For example, the finding that the aldolase activity of sensitive erythrocytes is higher than that of normal erythrocytes[30] may be due in part to the relative youth of the sensitive cells, if, as has been reported,[26] the activity of this enzyme decreases as the cells age. The relative importance of this factor as compared to the postulated compensatory increase cannot be assessed at this time.

The two conditions represent genetic variants expressed as a deficiency of G-6-PD. Kirkman[33, 34] was unable to detect qualitative differences in the erythrocyte G-6-PD of primaquine-sensitive Negroes even after concentrating the enzyme 10,000 fold. He has recently reported that in one Caucasian infant with CNSHA the erythrocyte G-6-PD was not only low, but also quantitatively different than the normal.[35] This finding suggests that different mutant genes are operative in the 2 states.

Several mutant genes or genetic modifiers may influence the activity of G-6-PD. Absence of an activator for G-6-PD has recently been reported by Rimon and associates[36] in primaquine-sensitive Sephardic Jews, in whom the hemolytic susceptibility is greater than that of primaquine-sensitive Negroes, but less than that of CNSHA. Probably more genetic variants expressed as a deficiency of G-6-PD will be found; it can be expected that the erythrocyte life span in these disorders will vary depending upon the severity of the defect.

Addendum. Subsequent to the studies reported here we have completed additional DFP^{32} erythrocyte survival curves on 2 normal men and one primaquine-sensitive man with confirmatory results. Erythrocyte survival curves with the DFP^{32} technique on 4 strongly expressed primaquine-sensitive Negro women also have shown a significant shortening of the red cell life span.

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The hemolytic effect of primaquine. XIII. Gradient susceptibility to hemolysis of primaquine-sensitive erythrocytes

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American Negroes whose erythrocytes have deficient glucose-6-phosphate dehydrogenase and diminished reduced glutathione undergo hemolysis following the ingestion of primaquine. If the same drug dosage is continued, the acute clinical hemolysis and anemia disappear. This self-limited anemia has been divided into 3 phases: (1) the acute hemolytic phase, (2) the recovery phase, and (3) the equilibrium, or "resistant" phase. This study established that the "resistant" phase is not absolute but relative; a second acute hemolysis, characterized by the same 3 phases as the first, can be induced during the "resistant" phase by an increase of the dosage. In addition, a subclinical but definite hemolytic process was demonstrated in the "resistant" phase. It is concluded that the susceptibility of primaquine-sensitive erythrocytes to hemolysis is not an all-or-none phenomenon; the cells have a graded susceptibility to hemolysis and become progressively more vulnerable as the cells age in vivo. The hemolytic gradient probably reflects the decrease in activity of glucose-6-phosphate dehydrogenase and, possibly, other enzymes during senescence of erythrocytes.

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Supported in major part by the Research and Development Command, Office of the Surgeon General, Department of the Army, under Contract DA-49-007-MD-566 with the Department of Medicine, University of Chicago, Alf. S. Alving, M.D., responsible investigator, and in part by Winthrop Laboratories, New York, N. Y.; Parke, Davis & Company, Detroit, Mich.; and Eaton Laboratories, Inc., Norwich, N. Y.

The investigations reported in this paper would not have been possible without the cooperation of the inmates and administrative staff of the Illinois State Penitentiary, Stateville Branch, Joliet, Ill.

A preliminary report of this investigation was published in J. Clin. Invest. 38: 1016, 1959.

Received for publication Nov. 16, 1960.

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Ten to 15 per cent of American Negro men experience acute hemolysis during the ingestion of primaquine,[1] nitrofurans,[2] phenacetin,[3] sulfonamides,[3] many other drugs, and some vegetable foods, e.g., fava beans.[4] Primaquine-sensitive erythrocytes are best characterized by deficient activity of glucose-6-phosphate dehydrogenase (G-6-PD).[5] Primaquine sensitivity, or, as it has been called, glucose-6-phosphate dehydrogenase deficiency, is an inborn error of metabolism transmitted by a sex-linked gene of intermediate dominance.[6-10] The trait is widely distributed throughout the world and, in general, the occurrence of primaquine-sensitivity parallels the distribution of falciparum malaria.[11, 12] The dark-skinned races are more frequently affected.[4, 8]

Dern and associates[13] observed that the administration of twice the therapeutic dose of the antimalarial drug, primaquine, induced a hemolytic anemia which is self-limited even though the drug dosage is continued. Beutler and coworkers[14] were able to show, by labeling erythrocytes of a narrow age range with Fe^{59}, that in primaquine-sensitive Negro men the erythrocytes 8 to 21 days old were not susceptible to hemolysis by 30 mg. of primaquine daily, yet erythrocytes 63 to 76 days old were vulnerable. They concluded that the younger erythrocytes were insensitive to primaquine because the cells which hemolyzed had been in the circulation for slightly more than one-half the life span of normal cells and because doubling the daily dose of primaquine after the older cells had been destroyed did not appear to alter complete hematologic recovery.[13] The studies in this report were planned to determine whether the circulating erythrocytes in primaquine-sensitive individuals fall into 2 distinct groups, those which are susceptible to hemolysis and those which are not, as proposed by Beutler and colleagues,[14] or whether all of the erythrocytes are susceptible, the older differing from the younger only in the degree of susceptibility. The second hypothesis implies a gradient hemolytic susceptibility as a function of red cell age. To evaluate these 2 hypotheses, an acute hemolysis was induced in several primaquine-sensitive Negro men* by the daily administration of 30 mg. of primaquine. This dosage was continued until clinical hematologic recovery was complete and, for a varying period of time during the subsequent "resistant" phase. The dosage was then increased several fold and observations were made for (1) evidence of a second acute hemolysis, and (2) evidence of chronic subclinical hemolysis during the "resistant" phase after apparent hematologic recovery.

Methods

The mean life span of erythrocytes was determined by the Cr^{51}† tagging technique[15] with some modifications.[16] Glucose-6-phosphate dehydrogenase activity was assayed in stroma-free hemolysate by coupling the reduction of oxidized triphosphopyridine nucleotide (TPN) by G-6-PD to the reduction of oxidized glutathione (GSSG) by glutathione reductase, as

*These investigations were carried out in healthy adult male volunteers. Susceptibility to hemolysis by primaquine was established by 2 criteria: (1) acute hemolysis following the ingestion of 30 mg. of primaquine for 14 days and (2) a deficiency of G-6-PD in erythrocytes. No primaquine-sensitive individuals received hemolytic drugs or donated blood during the 4 months preceding this study; no participant was deficient in iron. Dosages of primaquine were calculated on the base content of primaquine diphosphate and were administered orally.

†Rachromate, obtained from Abbott Laboratories, Inc., North Chicago, Ill.

described by Carson and associates[5] and Schrier and co-workers.[17] Reticulocyte counts were done on blood smears stained by a supravital technique with brilliant cresyl blue; preparations were permanent and no counterstaining was necessary. Each reticulocyte determination was based on the per cent of reticulocytes observed per 1,000 erythrocytes counted. The reduced glutathione (GSH) content of erythrocytes was determined in protein-free filtrates by a modification of the nitroprusside method.[18]

Results

Clinical course of a drug-induced hemolysis. The finding of Dern and colleagues[13] was confirmed; the daily administration of 30 mg. of primaquine to primaquine-sensitive Negro men produced a characteristic hemolysis with recovery, even though the same dosage of drug was continued (Fig. 1). It was also established that a second hemolysis, qualitatively and quantitatively identical to the first, occurred if the original dose was readministered after a drug-free interval of 4 months or longer. The self-limited hemolytic episode was arbitrarily divided into 3 phases: (1) the acute hemolytic phase, (2) the recovery phase, and (3) the equilibrium, or "resistant" phase.[13] The acute hemolysis was characterized by a rapid fall in the hematocrit level, a decrease in the GSH content of the erythrocytes,[18] and a fall in the catalase activity.[19] Anemia, increased reticulocytosis, hemoglobinuria, scleral icterus, and other clinical signs of hemolysis usually were not apparent until 2 to 4 days after the beginning

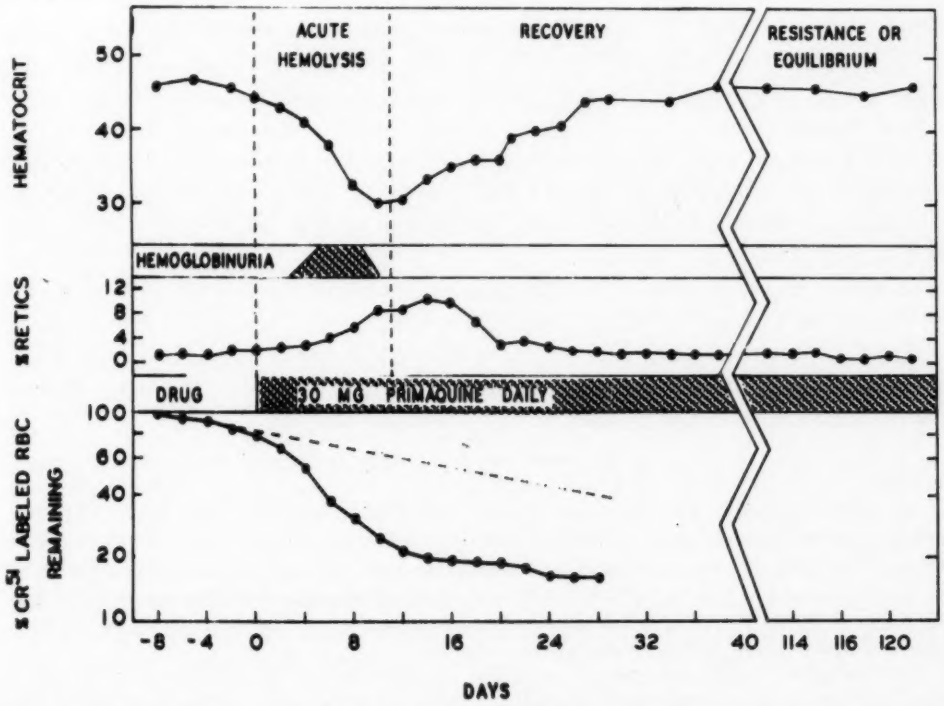

Fig. 1. The clinical course of an acute, self-limited hemolytic anemia induced by the administration of 30 mg. primaquine to drug-sensitive men. Composite data from 3 sensitive Negro men with comparable levels of G-6-PD deficiency. (Reprinted from Bull. World Health Organ. 22: 625, 1960.)

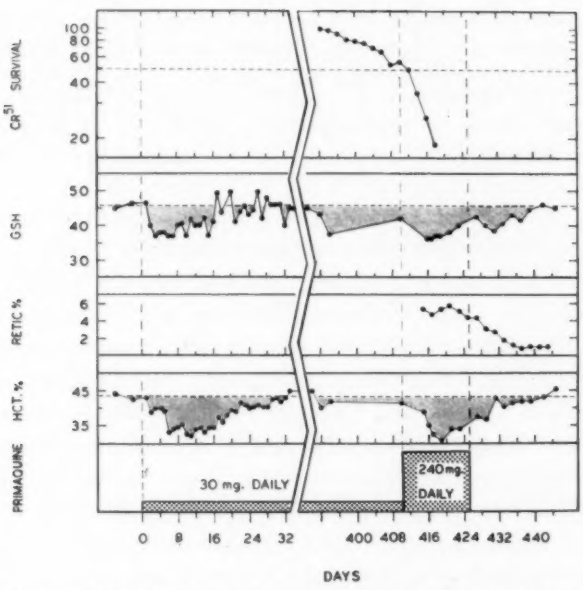

Fig. 2. Following a self-limited acute hemolytic anemia in a primaquine-sensitive Negro man receiving 30 mg. of primaquine daily, the dosage was continued for more than a year. During the resistant phase an increase in dosage induced a second acute hemolytic episode.

of primaquine administration. The recovery phase commenced with an increase in the reticulocyte count and was followed by the equilibrium, or "resistant" phase, which began when the hemoglobin and hematocrit returned to pre-drug control values.

The 6 primaquine-sensitive volunteers chosen for further investigation had previously demonstrated a comparable degree of hemolytic susceptibility to primaquine. Each manifested major expression of the genetically transmitted trait among American Negroes.

Relative susceptibility of erythrocytes to hemolysis during the recovery and the "resistant" phase. Detailed hematologic studies were performed in 3 of the 6 primaquine-sensitive volunteers to whom varying doses of primaquine were administered during the "resistant" phase. The time at which the drug dosage was increased was varied in each of the following studies.

1. The clinical course of 2 successive hemolytic episodes induced during the prolonged administration of primaquine to a sensitive Negro man is shown in Fig. 2. He received 30 mg. of primaquine daily for *more than 1 year* after clinical recovery from the initial self-limited hemolytic anemia before the daily dose of primaquine was increased. When the dose was raised to 240 mg., he experienced a second acute hemolytic episode which, like the first, was associated with a fall in the hematocrit and in the GSH content of the erythrocytes and was followed by an increase in reticulocytosis during recovery. The high dosage of primaquine was discontinued before recovery was complete.

2. The second drug-sensitive male volunteer received 30 mg. of primaquine daily for *5 weeks.* When the hematocrit had returned to normal and the GSH to pre-drug levels, the primaquine dosage was increased to 240 mg. daily and he experienced a second clinical hemolysis. This hemolysis was qualitatively the same as the initial one; however, the severity of the second hemolysis induced by the eightfold increase of primaquine was not as great as in the initial hemolysis (Fig. 3).

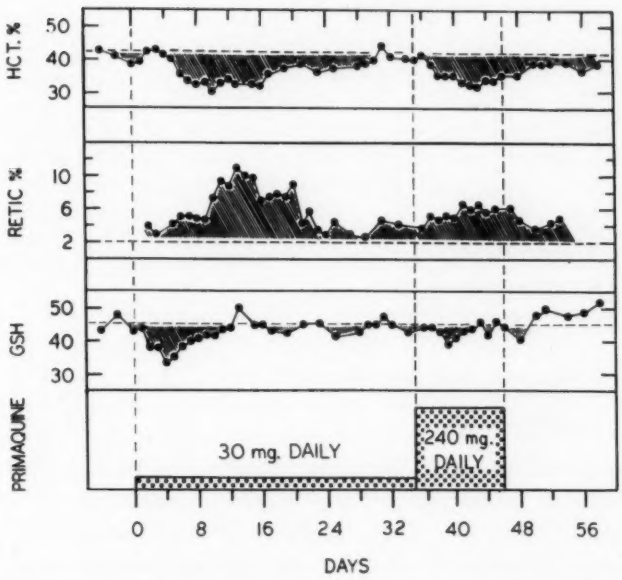

Fig. 3. Following a self-limited hemolytic anemia in a primaquine-sensitive Negro man receiving 30 mg. of primaquine daily, the dosage was continued for 5 weeks, at which time a second hemolysis was induced by raising the dose to 240 mg. daily.

3. The third primaquine-sensitive individual also initially was given 30 mg. of primaquine daily. *As soon as* it was possible to establish the beginning of hematologic recovery (on the sixth day of drug administration)* the daily dosage was increased to 120 mg. daily. The increase in the drug dosage interrupted recovery, and further hemolysis ensued.

Chronic hemolysis during the "resistant" phase. The effect of prolonged administration of progressively greater dosages of primaquine on the life span of erythrocytes and on erythropoiesis was studied in the remaining 3 drug-sensitive Negro volunteers. In each individual the hematocrit, erythrocyte life span (Cr^{51} tagging technique), and reticulocyte count were determined under physiological conditions during a 4 month control period prior to drug administration. After control values had been established, each volunteer was given 60 mg. primaquine base daily for 4 months, whereupon the daily dose was raised to 120 mg. for a second consecutive 4 month period. During the last 3 weeks of each 4 month period of drug administration the parameters studied during the control period (life span of the erythrocytes, hematocrit and reticulocyte count) were re-evaluated. Four months after primaquine administration was stopped, final evaluation of 2 parameters (hematocrit and reticulocyte count) were again made after complete hematologic recovery.

The life span of the erythrocytes in these 3 primaquine-sensitive Negro men was slightly shorter than normal (Cr^{51} half life: 23, 25, and 26 days) even in the absence of hemolytic drugs (Table I), which is in agreement with the observations of Brewer and associates[20] indicating that under physiologic conditions a mild but compensated subclinical hemolysis was present. During the late

*At this time there was no further fall in hematocrit; a rise in the GSH content and a stabilization or decrease in the reticulocyte count had occurred.

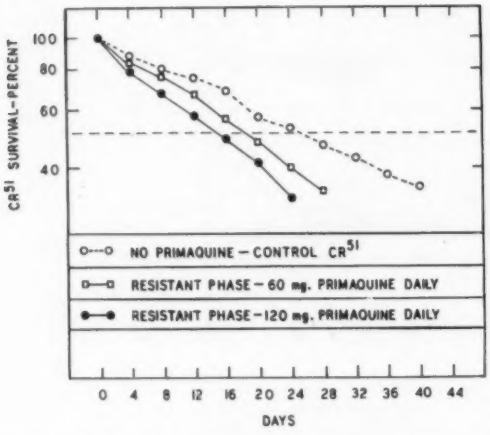

Fig. 4. Life span studies of erythrocytes from a primaquine-sensitive Negro man during the late resistant phase associated with 60 and 120 mg. primaquine base daily. Similar results were obtained in 2 other sensitive Negro men.

"resistant" phases associated with the administration of 60 and 120 mg. primaquine daily, subclinical hemolysis of increasingly greater severity could be detected (Fig. 4 and Table I). The degree of chronic hemolysis during the resistant phase, as determined by the increase in reticulocyte counts and the shortened life span of the erythrocytes (Fig. 3), was directly proportional to the dosage of primaquine. During the "resistant" phase associated with each dosage the hematocrit remained normal due to the slight but significant increase in the rate of erythropoiesis.

IV. The administration of large doses of primaquine to nonsensitive men. The administration of large doses of primaquine to nonsensitive men did not induce clinical hemolysis. However, slight decreases in the hematocrit, in the GSH content of the erythrocytes, and in the life span of the erythrocytes, as well as a slight increase in the reticulocyte count was observed in some nonsensitive men receiving high dosages of primaquine (Fig. 5). These changes were consistent with a mild hemolysis; however, alterations secondary to removal of blood samples for biochemical studies, or changes secondary to

Table I. Evidence of chronic hemolysis during the fourth month of consecutive "resistant" phases in one primaquine-sensitive Negro man*

Consecutive 4 month periods	*Life span Cr^{51} half life*	*Reticulocytes (%)*		*Hematocrit mean*
		Mean†	*± S. D.*	
Control period (no primaquine)	23	1.2 ± .27	(12)‡	43
Resistant phase (primaquine 60 mg. daily)	19	1.8 ± .54	(17)	43
Resistant phase (primaquine 120 mg. daily)	15	2.4 ± .66	(27)	43
Control period (no primaquine)	not done	1.0 ± .14	(6)	46

*Similar results were obtained in 2 other primaquine-sensitive Negro men.
†The difference between the means of the reticulocyte counts were statistically significant (p value > .001).
‡Figures in parentheses refer to the number of determinations.

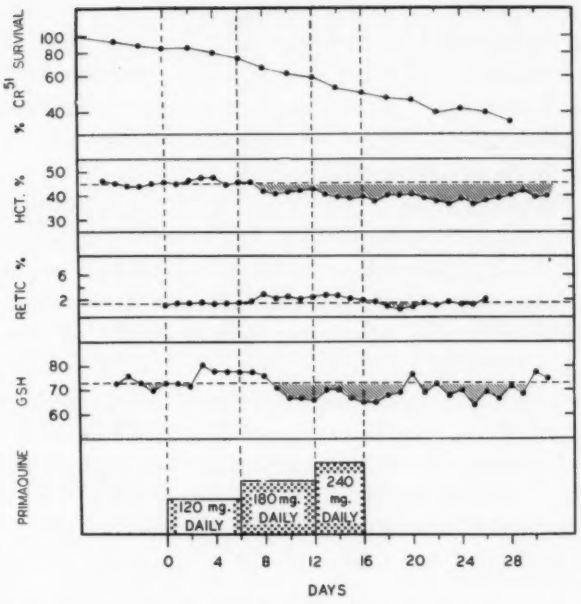

Fig. 5. Hematologic parameters during the administration of large dosages of primaquine to a Negro man with normal hemolysate G-6-PD activity.

methemoglobinemia which occurred with primaquine administration might also play a role. Daily administration of dosages of up to 240 mg. of primaquine to individuals with normal hemolysate G-6-PD activity certainly did not result in hemolysis of clinical importance.

Discussion

A "resistant" or equilibrium phase follows the recovery from acute hemolytic anemia produced by the destruction of the older erythrocytes during the administration of 30 mg. of primaquine to drug-sensitive Negro men.[13, 14] However, the resistance of the erythrocytes to further hemolysis is relative and not absolute, since a second hemolysis can be induced during the "resistant" phase by an increase in the dose of primaquine. The second hemolysis can be superimposed on the first if the dose of drug is increased during reticulocytosis and recovery, or it may be precipitated by suddenly increasing the drug dosage after the "resistant" phase has been maintained for 4 to 12 months by the continuous administration of a smaller dose of drug. This continued hypersusceptibility to hemolysis during the "resistant" phase is consistent with the reported findings[18] of a positive Heinz body test, positive glutathione stability test, and low G-6-PD activity of primaquine-sensitive erythrocytes during the "resistant" phase.

The "resistant" phase, in addition, is characterized by continued mild hemolysis, fully compensated by a slight increase in erythropoiesis; a normal hematocrit level is maintained as long as the severity of the hemolysis does not exceed the ability of the bone marrow to respond. The severity of the subclinical

hemolysis during the equilibrium phase is dependent on the dosage of drug. Dern and colleagues[13] were not able to demonstrate either a decrease in the life span of primaquine-sensitive erythrocytes or any susceptibility to further acute hemolysis during the "resistant" phase due to a failure to incorporate certain experimental precautions, the need for which was not apparent at the time of their investigations. With the recognition of the necessity of these precautions, as well as further standardization of methods, the earlier finding of a normal erythrocyte life span during the "resistant" phase has to be modified.

Primaquine-sensitive erythrocytes are deficient in G-6-PD,[5] an enzyme which decreases during in vivo aging.[21, 22] The G-6-PD deficiency limits the rapid regeneration of TPNH and GSH essential for the reduction of redox intermediates which would otherwise inactivate vital cellular components.[23] Primaquine, and many other similarly hemolytic compounds, are or can be transformed into redox intermediates[24, 25] capable of depleting TPNH and GSH. The younger erythrocytes present in the equilibrium phase have a higher level of G-6-PD* activity and a greater ability to regenerate TPNH; this may account for the self-limited nature of the hemolysis and explain the relative resistance to hemolysis during continued administration of primaquine.

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*Although Flanagan and associates[18] previously reported the G-6-PD activity of primaquine-sensitive erythrocytes to be equal before primaquine administration and during the "resistant" phase, the difference might not have been apparent with the techniques used at that time. It will be necessary to statistically evaluate a large number of G-6-PD activity determinations in order to distinguish any difference.

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Electrolyte, nitrogen, and water content of human leukemic leukocytes: Relation to cell maturity

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The water, solids, nitrogen, ash, sodium, potassium, calcium, and magnesium content of human leukocytes in the blood of patients with various types of leukemia has been determined. The mean content and its standard error per 10^{12} leukocytes for each constituent, expressed in grams for the first 4 items and in milliequivalents for the others, in the order listed above, are as follows: Chronic granulocytic leukemia: 449 ± 14, 126 ± 4, 16.2 ± 0.6, 7.7 ± 0.2, 27.7 ± 2.2, 49.3 ± 1.9, 0.9 ± 0.1, 8.0 ± 0.4, respectively; Chronic lymphocytic leukemia: 215 ± 14, 54 ± 4, 7.2 ± 0.4, 4.5 ± 0.2, 17.8 ± 2.2, 23.0 ± 3.5, 0.3 ± 0.03, 3.5 ± 0.2, respectively. The differences in the corresponding values for the two types of leukemia are statistically significant. A small number of determinations on other types of leukemia gave values within the range of those found for leukocytes in the blood of patients with chronic granulocytic and lymphocytic leukemia. A multiple regression analysis revealed that even leukocytes of the same series differ in their content according to size and/or maturity of the cells. In the granulocytic series, water, potassium, and sodium remain unaltered with the age of the cell, magnesium decreases, and total solids, nitrogen, and calcium increase, in contrast to the lymphocytic series, in which no change in the potassium but marked decrease in every other constituent occurs with cell maturity.

The biochemical and biophysical effects of electrolytes and the profound consequences following their alteration are well recognized.[1-4] Leukocytes are exceptionally suitable for studies on cellular metabolism and composition, as they can be separated in pure form, particularly from leukemic blood, by simple

From the Division of Experimental Medicine, University of Oregon Medical School, Portland, Ore.
Supported by Grant No. CY-3374 from the United States Public Health Service, Grant No. AT(45-1)-581 from the United States Atomic Energy Commission, and by the Medical Research Foundation of Oregon.
Received for publication Jan. 6, 1961.
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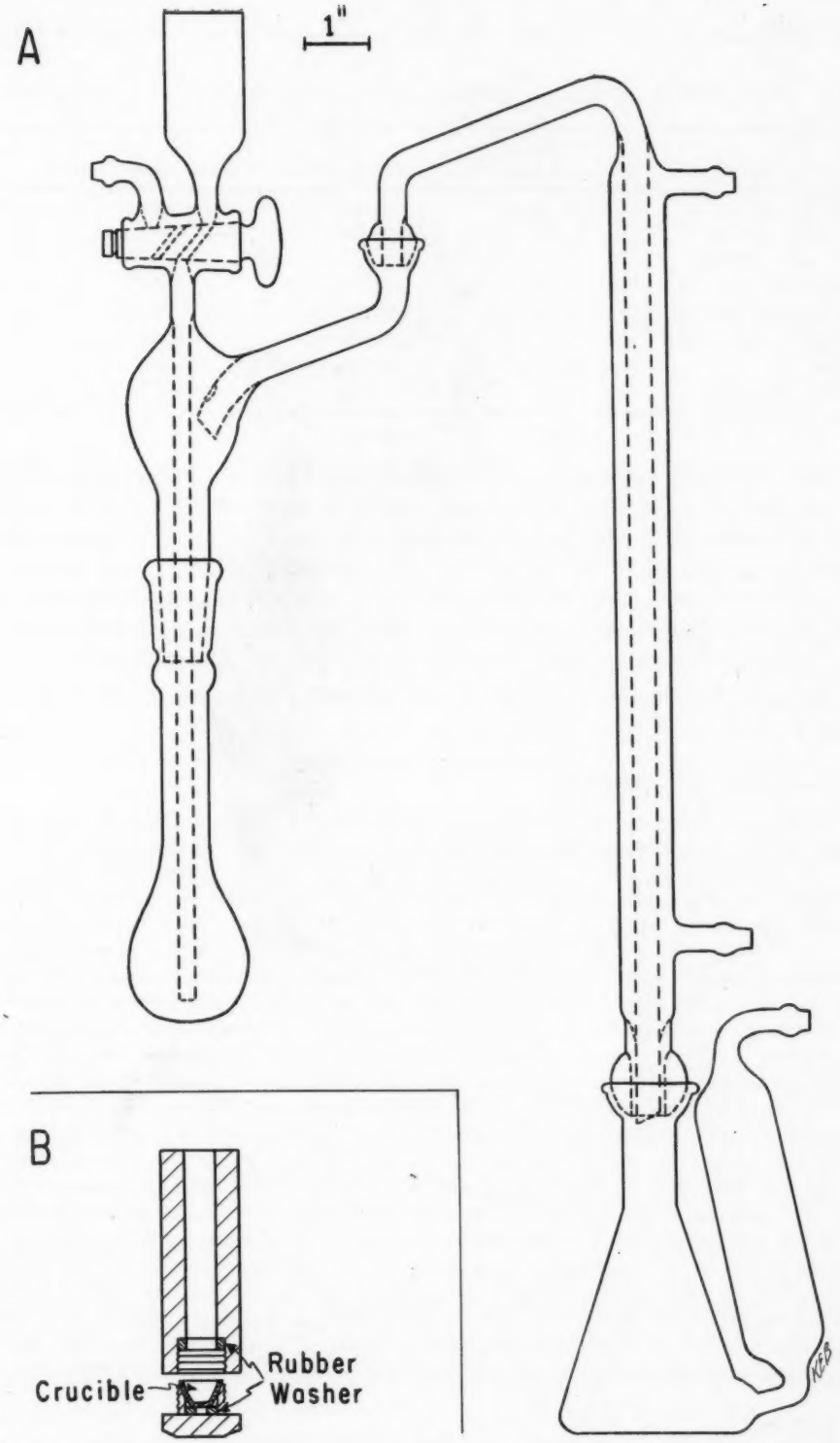

Fig. 1. A, Micro-Kjeldahl distillation apparatus. *B,* Special centrifuge tube consisting of 2 Lucite parts. The tube is assembled by screwing the lower part tightly into the upper, with the microcrucible between the parts.

Table I. Composition of human leukemic leukocytes: content per 10^{12} leukocytes

Type of leukemia	*Number of analyzed samples*	*Water (Gm.)*	*Total solids (Gm.)*	*Total nitrogen (Gm.)*
Chronic granulocytic	51	449 ± 14	126 ± 4	16.2 ± 0.6
Chronic lymphocytic	29	215 ± 14	54 ± 4	7.2 ± 0.4
Acute and subacute monocytic	9	428 ± 38	108 ± 9	14.9 ± 0.9
Acute lymphocytic	6	320 ± 48	70 ± 7	10.2 ± 1.3
Subacute lymphocytic	2	392	88	11.5
Plasmocytic	2	693	170	21.3
Chronic eosinophilic	1	548	179	23.0

methods. Leukocytes almost entirely of the same series (granulocytic, lymphocytic, monocytic, etc.) but varying in maturity can be obtained from patients with leukemia. Elucidation of differences in composition that may exist between leukocytes of different series or of changes that may occur during the maturation of leukocytes is thus possible, as was previously demonstrated in a study of the phosphorus compounds.[5] This paper shows that human leukemic leukocytes of different series differ in their electrolyte, nitrogen, and water content and that marked changes in composition occur during the maturation of leukocytes.

Materials and methods

Leukocytes were isolated from oxalated blood samples obtained from 53 patients with various types of leukemia, using purified phytohemagglutinin[6] as previously described.[5] The isolated leukocytes were counted in their final suspension in plasma with the use of a hemacytometer. The leukocytes of each sample were predominantly of one series (97 to 99 per cent) containing only 1 to 2 erythrocytes per 100 leukocytes. The per cent of immature cells, i.e., cells with nucleoli (myeloblasts, progranulocytes, and early myelocytes) in granulocytic leukemia, and the lymphoblasts plus prolymphocytes in lymphocytic leukemia, was established by differential counts on at least 500 leukocytes. The leukocyte volume was determined by centrifugation to constant volume.[7] Wet weight determinations were done by weighing a known number of packed leukocytes, centrifuged without washing, into a preweighed microcrucible which served as the bottom of a specially designed centrifuge tube (Fig. 1, *B*). The trapped plasma in the packed leukocytes is negligible.[7] Total solids were determined by lyophilization of the leukocytes in the microcrucibles, followed by drying to constant weight at 100° C. in vacuum. Total nitrogen was determined on the dried leukocytes by the micro-Kjeldahl procedure.[8] An all-glass distillation apparatus was used to distill the ammonia directly from the digestion flask (Fig. 1, *A*). Ash was determined by ashing the dried leukocytes in microcrucibles at 500° C. in a muffle furnace. The ash was dissolved in 1.0 N HCl and used to determine Na, K, and Ca by flame spectrophotometry[9] at 589.5, 768, and 423 mμ emission lines, respectively, and Mg, by the Titan yellow method.[10] The Beckman DU spectrophotometer with a photomultiplier attachment and a hydrogen-oxygen microburner was used. Corrections for the erythrocytes in each sample were made and found to be negligible. Statistical analysis of the results was preformed as previously described.[5]

Results

Complete duplicate to quadruplicate analyses were performed for each constituent on each of 100 samples and the results were expressed as content per 10^{12} leukocytes.

Ash (Gm.)	*Sodium (mEq.)*	*Potassium (mEq.)*	*Calcium (mEq.)*	*Magnesium (mEq.)*
7.7 ± 0.2	27.7 ± 2.2	49.3 ± 1.9	0.9 ± 0.1	8.0 ± 0.4
4.5 ± 0.2	17.8 ± 2.2	23.0 ± 3.5	0.3 ± 0.03	3.5 ± 0.2
7.7 ± 0.9	27.8 ± 4.4	48.9 ± 6.0	0.7 ± 0.1	8.4 ± 1.6
5.7 ± 0.5	31.0 ± 6.3	22.7 ± 10.2	0.7 ± 0.2	6.4 ± 1.0
6.3	41.1	26.0	0.5	7.2
14.1	78.6	44.6	1.5	14.5
7.4	23.0	44.8	1.2	5.8

Composition of leukocytes of various leukocytic series. Results from patients with the same type of leukemia were pooled and found to be normally distributed ($P<0.05$). Means and standard errors of the means were computed and are shown in Table I. The content per kilogram of leukocytes or per kilogram of leukocyte water can be calculated from the data in this table. Leukocytes from patients with chronic lymphocytic leukemia were found to contain significantly lower amounts of each constituent per cell than leukocytes from patients with granulocytic leukemia ($P<0.001$). On a wet weight basis leukocytes of the granulocytic series were not significantly different ($P>0.05$) in their N, K, and Mg content from those of the lymphocytic series, but they were

Table II. Relation of composition to maturity of human leukemic leukocytes

Constituent	*Regression equation**	*Predicted values per 10^{12} leukocytes*	
		Immature	*Mature*
Granulocytic series			
Water (Gm.)	$\hat{Y} = 59 + 0.742X_2$	449 ± 14	449 ± 14
Total solids (Gm.)	$\hat{Y} = 39.6 - 0.65X_1 + 0.158X_2 + 0.336X_3$	75 ± 8	140 ± 3
Total nitrogen (Gm.)	$\hat{Y} = 4.5 - 0.06X_1 + 0.025X_2$	11.3 ± 1.6	17.6 ± 0.5
Ash (Gm.)	$\hat{Y} = 1.2 - 0.007X_2 + 0.056X_3$	7.7 ± 0.2	7.7 ± 0.2
Sodium (mEq.)	$\hat{Y} = 8 + 0.114X_2 - 0.808X_3$	27.7 ± 2.2	27.7 ± 2.2
Potassium (mEq.)	$\hat{Y} = 13 + 0.092X_2 - 0.487X_4$	49.3 ± 1.9	49.3 ± 1.9
Calcium (mEq.)	$\hat{Y} = 0.05 - 0.01X_1 + 0.038X_4$	0.11 ± 0.1	1.11 ± 0.1
Magnesium (mEq.)	$\hat{Y} = 1.4 + 0.05X_1 + 0.016X_2 - 0.103X_4$	11.9 ± 1.6	6.9 ± 0.5
Lymphocytic series			
Water (Gm.)	$\hat{Y} = -13.3 - 1.26X_1 + 0.830X_2$	496 ± 26	162 ± 6
Total solids (Gm.)	$\hat{Y} = 11.6 - 0.37X_1 + 0.180X_2$	113 ± 8	50 ± 2
Total nitrogen (Gm.)	$\hat{Y} = 1.7 - 0.04X_1 + 0.024X_2$	16.1 ± 0.9	6.7 ± 0.2
Ash (Gm.)	$\hat{Y} = 2.1 + 0.007X_2 + 0.027X_3$	8.1 ± 0.4	4.2 ± 0.3
Sodium (mEq.)	$\hat{Y} = -8.1 - 0.3X_1 + 0.118X_2$	52.2 ± 6.2	16.8 ± 1.4
Potassium (mEq.)	$\hat{Y} = \overline{Y} = 23.1$	23.1 ± 3.5	23.1 ± 3.5
Calcium (mEq.)	$\hat{Y} = 0.34 + 0.001X_2 - 0.014X_3$	0.8 ± 0.1	0.23 ± 0.1
Magnesium (mEq.)	$\hat{Y} = 1.30 + 0.010X_2$	9.0 ± 1.0	3.4 ± 0.3

*$\hat{Y}$ is the predicted constituent; X_1 is the percentage of immature leukocytes; X_2 is the cell volume in milliliters per 10^{12} leukocytes; X_3 and X_4 are the K and Na content, respectively.

found to contain less water, ash, and Na and more total solids and Ca ($P<0.001$). The same relationship was observed when the electrolyte content was expressed on the basis of leukocyte water. Definite conclusions cannot be drawn, in respect to leukocytes, from other types of leukemia, although the small number of determinations gave values within the range of those for chronic lymphocytic and granulocytic leukemia.

Relationship of composition to maturity and size of leukocytes. The wide variation in leukocyte composition, maturity, and size observed among samples of leukocytes from patients with the same type of leukemia suggested a possible correlation of these factors. Multiple regression analyses for each constituent were therefore done, using as predictors only variables which showed statistically significant partial regression coefficients ($P<0.01$).

GRANULOCYTIC SERIES. The volume of these cells was found to be 526 ± 17.5 ml. (mean ± standard error) per 10^{12} leukocytes and independent of their maturity. However, every constituent was found to depend on one or more predictors. Regression equations were derived for each constituent and are listed in Table II. The following relationships are indicated by these equations. The amount of every constituent except Ca, which was independent of cell volume, increased as the cells increased in size. Total solids and ash increased with increased K content, the latter being inversely related to the Na content. Immature leukocytes of this series contained less total solids, N, and Ca and more Mg than mature leukocytes of comparable size, but there was no difference in their water, ash, Na, and K content. Table II shows the mean content (plus or minus its standard error) of immature and mature leukocytes as predicted by the regression equations. It can be seen that the mature leukocytes contained about twice the amount of total solids, 50 per cent more N, 10 times as much Ca, and half as much Mg as did the immature leukocytes.

LYMPHOCYTIC SERIES. The combined data from chronic, acute, and subacute lymphocytic leukemias were analyzed. In this case the volume of the cells, $\hat{Y}$, was found to be correlated with the per cent of immature cells, X, and given by the equation

$$\hat{Y} = 211 + 5.54X,$$

which predicts the mean volume of immature and mature leukocytes as 765 and 211 ml. per 10^{12} cells, respectively. The regression equations for each constitutent are listed in Table II and indicate the following. The K content was constant, while every other constituent increased with cell volume. Ash increased and Ca decreased with the K content. The water, total solids, N, and Na content per unit volume of cells was lower in the immature than in the mature leukocytes. From the predicted mean content and standard error (Table II) it can be seen that the immature leukocytes contained 2 to 3 times more of each constituent than mature leukocytes of this series, except K, for which no significant difference was found.

The predicted value for each constituent in the granulocytic series was compared with the corresponding value in the lymphocytic series by the "t" test at the 1 per cent level of significance. Immature cells of the lymphocytic

series were found to contain significantly higher amounts of total solids, N, Na, and Ca, and less K and Mg than immature cells of the granulocytic series, but lower amounts of total solids, K, and Ca, and more Na than mature cells of the granulocytic series. Mature lymphocytes contained significantly lower amounts of every constituent than either immature or mature cells of the granulocytic series.

Discussion

Several investigators have used leukocytes from various sources in studies of cellular metabolism and composition. Hoppe-Seyler[11] analyzed cells of dried pus. Others have used leukocytes from peritoneal exudates of rabbits[12-17] and guinea pigs,[18] and from venous blood of horses[19-21] and dogfish.[22] Only a few determinations of total nitrogen,[23] water, nitrogen, sodium, and potassium[24, 25] have been performed on human leukocytes. The reported values are not significantly different from the values presented here. A high sodium to potassium ratio in animal and human leukocytes, as compared to that of erythrocytes, brain, and muscle is evident from our results and from data in the literature. This may be due to pinocytosis, which leukocytes exhibit in vitro,[26] and/or to a less efficient sodium exclusion mechanism.

The statistical analysis of our data shows that leukocytes of different series or of the same series but of different maturity differ in their water, nitrogen, and electrolyte composition. The difference in cell size contributes heavily to the observed differences in cellular content, but fails to explain them completely, as not all of these differences are nullified by expressing the data per milliliter or per kilogram of leukocytes. Clear-cut differences between leukocytes of the granulocytic and the lymphocytic series were thus demonstrated not only in their composition but also in the direction and magnitude of changes in composition accompanying their maturation. The marked biochemical changes accompanying alterations in electrolytes[1-3] suggest that some of the metabolic characteristics of leukocytes may be partly due to their distinctive electrolyte composition. The electrolyte changes which accompany maturation may be of importance in metabolic alterations that lead to loss of the ability of the cells to divide. In this regard, it is perhaps significant that magnesium which is required by many enzymes, including those concerned with nucleic acid synthesis, was found in much higher amounts in the immature than in the mature leukocytes.

It should be emphasized that the values given here for leukemic leukocytes may differ from those of normal human leukocytes. Human malignant tissues have been found to contain more water, potassium, and other electrolytes than the corresponding normal tissues,[25, 27] but the relative immaturity of cells and higher cellularity of malignant tissues may be in part responsible for these differences.

We are grateful to Drs. Edwin E. Osgood, Arthur J. Seaman, Robert D. Koler, and Bernard Pirofsky for making available their patients for this study, and to the Western Data Processing Center, University of California at Los Angeles, for the electronic processing of the data.

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Ultracentrifugal fractionation of human erythrocytes on the basis of cell age

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An ultracentrifugal technique for the fractionation of human erythrocytes according to cell age is described. The method utilizes the Spinco Model L preparative ultracentrifuge, which is equipped with a swinging bucket rotor. The erythrocytes are centrifuged in Lusteroid tubes for 2 hours at 67,000 g; then the erythrocyte column is divided into layers by a specially designed tube cutter. A comparison of this method with that of ordinary centrifugation on 14 blood samples obtained at intervals from a normal human male following administration of a tracer dose of Fe^{59} demonstrated that ultracentrifugation represents a marked improvement in terms of stratification of erythrocytes according to cell age.

Following Key's[1] observation that reticulocytes have a lower specific gravity than mature erythrocytes, simple centrifugation has been used extensively, particularly in recent years, for the fractionation of erythrocytes according to age.[2-16] However, this simple method yields fractions which are not pure in respect to cell age. We observed that separation of erythrocytes by age improved as the time of centrifugation and the centrifugal force increased. This led us to use ultracentrifugal fields to obtain remarkably better separation of erythrocytes by age.

Materials and methods

Blood samples were obtained at intervals by venipuncture from a normal man following intravenous injection of a tracer dose of Fe^{59} as ferrous citrate. Heparin was used as an anticoagulant. Fractionation of erythrocytes according to age was done by ultracentrifugation

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Supported by United States Atomic Energy Commission Contract AT(45-1)-581, by United States Public Health Service, National Cancer Institute Grant No. CY-3374, and by the Medical Research Foundation of Oregon.
Received for publication Jan. 6, 1961.

and the results were compared with those obtained by ordinary centrifugation at 1,600 g for 2 hours.[11] The Spinco Model L preparative ultracentrifuge with a swinging bucket rotor, type SW25.1 was used. The swinging buckets were equipped with nylon adapters to accommodate 0.5 by 2.5 inch Lusteroid tubes. In order to get as many erythrocytes as possible into these tubes and still take advantage of the distribution by age which occurs as they sediment through the plasma, 0.5 by 3.5 inch Lusteroid tubes were filled with blood

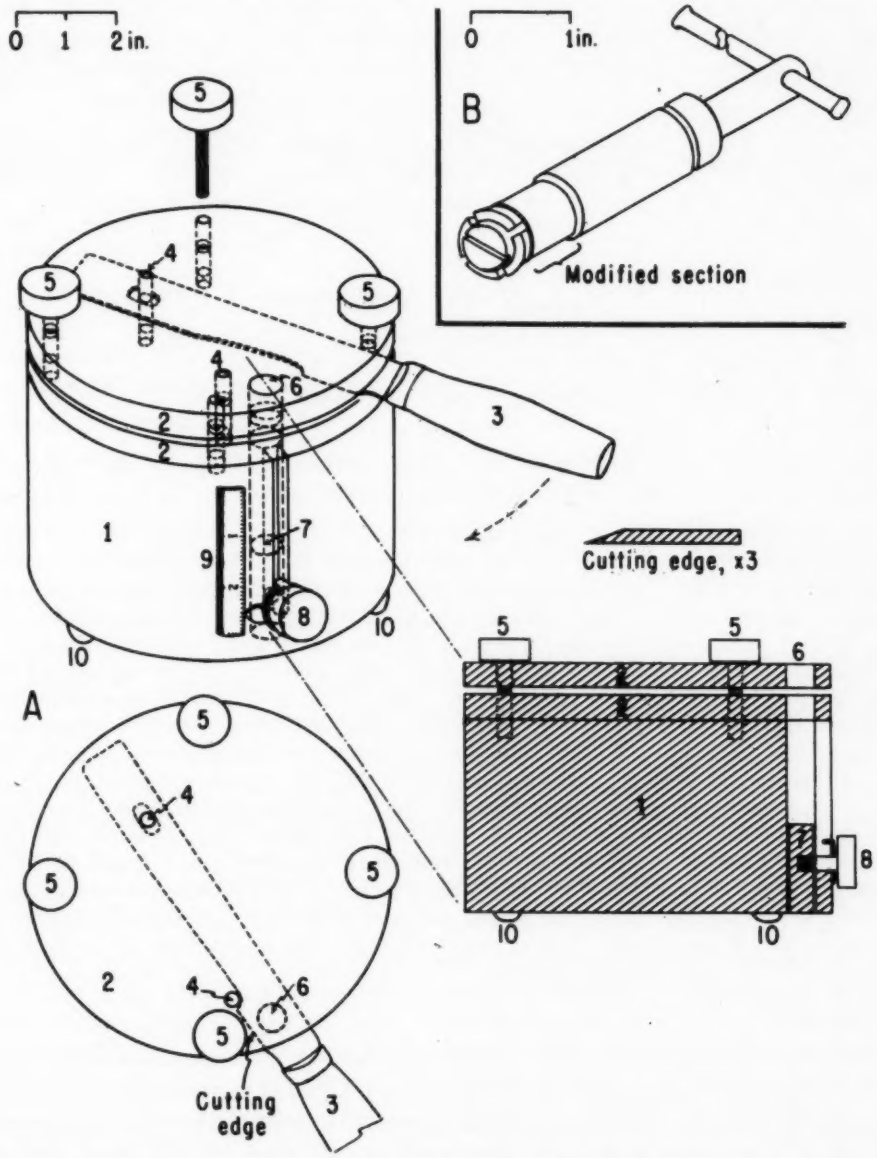

Fig. 1. *A*, Tube cutter: *1*, aluminum block; *2*, Plexiglass discs (the lower permanently set, the upper removable); *3*, cutting knife; *4*, permanently set brass pins serving as the fulcrum and the stop of the knife blade; *5*, securing thumbscrews; *6*, hole into which plastic tube fits snugly; *7*, brass plunger supporting tube, positioned and secured by thumbscrew (*8*); with point indicator of position on scale (*9*); *10*, supporting rubber feet. *B*, Knurl-tite wrench modified as shown.

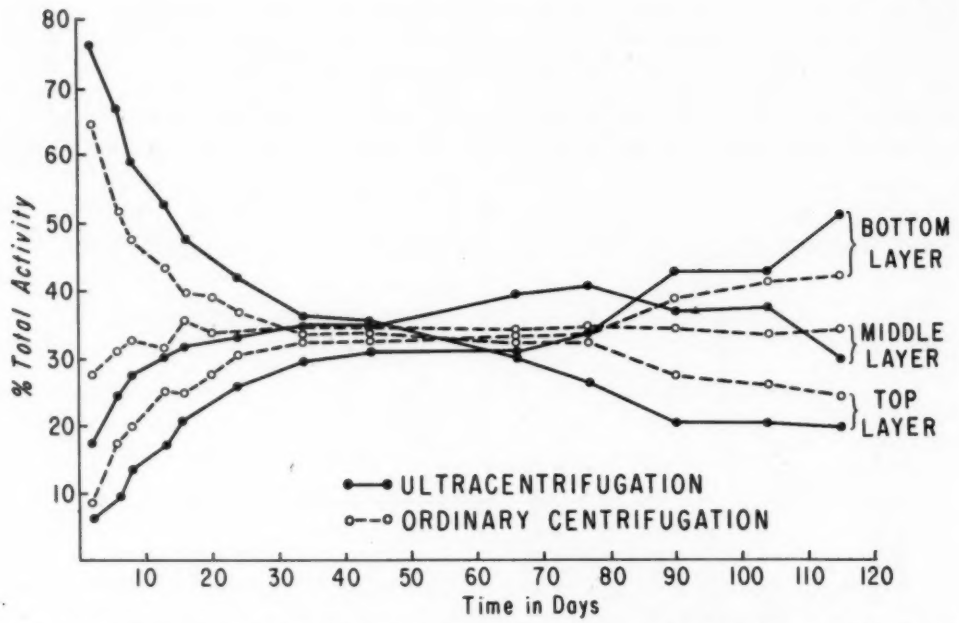

Fig. 2. Separation of erythrocytes by age using ordinary centrifugation at 2,000 g and ultracentrifugation at 90,000 g following in vivo labeling with Fe^{59}.

and centrifuged briefly in an International refrigerated centrifuge. Following removal of the supernatant plasma and buffy coat, the tubes were cut to 2.5 inches with a specially designed tube cutter (Fig. 1, *A*) and ultracentrifuged at 4° C. and 22,500 r.p.m. (67,000 g at the tip of the tube) for 2 hours. To avoid mixing, the rotor was allowed to coast to a complete stop. The Lusteroid tubes adhered so tightly to the nylon adapters following ultracentrifugation that removal was difficult. Lubrication or powdering of the adapter with talcum powder caused the Lusteroid tube to collapse during ultracentrifugation because the supporting frictional force which develops between adapter and tube is largely eliminated. A wrench* (Fig. 1, *B*) was therefore used to remove the tubes without undue difficulty. Trimming the end of the tightening sleeve to 20 mm. outside diameter (Fig. 1, *B*) permitted a better grasp of the tube. The erythrocyte column was divided into 3 equal layers by cutting transversely through the tube with the tube cutter. The blade of the knife served as the dividing partition while the erythrocytes above it were withdrawn with a syringe fitted with a 13 gauge blunt needle prior to raising the tube to cut another layer. The Fe^{59} present in each layer was determined with a well-type scintillation detector in conjunction with a pulse height analyzer and scaler and was expressed as per cent of the total Fe^{59} activity found in the 3 layers.

Results

Fourteen blood samples were withdrawn from a normal male subject over a period of 115 days after the administration of 8.5 μc of Fe^{59}; duplicate samples were fractionated by ordinary and ultracentrifugation. The Fe^{59} present in each layer was determined and expressed as per cent of the total activity found in the 3 layers. The results (Fig. 2) have been plotted against the time at which the samples were obtained. Both techniques of fractionation clearly show immediate incorporation of Fe^{59} into the reticulocytes which are concentrated in the top layer.[5, 7] The continuous change of specific gravity of

*The 5/8 inch Knurl-tite wrench (No. 2583) was made by Walsco Co., Rockford, Ill.

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aging erythrocytes which, upon centrifugation, results in stratification according to age, as judged by the gradual shift of Fe^{59} from top to bottom,[11] is also demonstrated by both methods. However, comparing the 2 techniques in respect to the distribution of the labeled cells among the 3 layers, it can be seen (Fig. 2) that during the early part of this study a greater portion of labeled erythrocytes congregated in the top layer when ultracentrifuged than when submitted to ordinary centrifugation and that the opposite is true for the middle and bottom layers. Midway through the study, ultracentrifugation yielded relatively more labeled cells in the middle layer and, during the last part of the study, relatively more in the bottom layer than did ordinary centrifugation. It is thus apparent that ultracentrifugation consistently resulted in far better stratification of the erythrocytes by cell age.

Discussion

The importance of the metabolic changes which accompany the aging of erythrocytes has stimulated the development of various methods of fractionating erythrocytes according to age. Thus, serial osmotic hemolysis[17] and differential agglutination separating transfused donor cells from those of the recipient[18] have been used.

The first use of the variation in specific gravity of erythrocytes was made by Bass and Johns,[19] who demonstrated that erythrocytes containing malarial parasites are concentrated in the top cell layer of centrifuged blood. Thirty years passed before it was realized that these cells are young erythrocytes.[20] Simple sedimentation[1, 3] and ordinary centrifugation[2, 4-10] had been used for many years to obtain erythrocytes rich in reticulocytes. However, it was not until a study was made of the distribution of Fe^{59}-tagged erythrocytes by centrifugation[11] that the progressive increase of specific gravity of the cells as they age was recognized and full advantage taken of this phenomenon. From the data presented here it is apparent that the older, denser erythrocytes can be driven through the mass of packed erythrocytes under the influence of ultracentrifugal fields, thus achieving a markedly improved stratification according to age. However, complete stratification of the labeled cells into a single layer was not achieved. The high centrifugal force has no apparent effect on the morphology of the erythrocytes. Use of the special tube cutter (Fig. 1, *A*) prevents mixing of erythrocytes in different layers, which occurs on simple pipetting, and permits metabolic studies on intact erythrocytes, which is impossible when the R.B.C. are frozen and then cut. This is also a distinct advantage over fractionation by serial osmotic hemolysis[17] which yields no intact erythrocytes. Ultracentrifugation is superior to differential agglutination[18] as it yields fractions of cells which are devoid not only of younger cells, as is the case with the latter method, but also of older erythrocytes.

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Studies concerning the turnover of fibrinogen I^{131} in the dog

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Iodinated dog fibrinogen was infused into normal dogs and the fibrinogen I^{131} of each was measured as the amount of radioactivity clottable by thrombin or precipitatable at 56° C. The half-life was 2.4 days. Fibrinogen was rapidly distributed between intra- and extravascular compartments, being demonstrable in the lymph by 30 minutes after infusion. Equilibration, as determined by the change in slope of the disappearance curve, was reached in approximately 24 hours; at this point 66 per cent of the infused fibrinogen I^{131} remained in the plasma. Anticoagulants did not effect fibrinogen survival time. A fibrinolytic agent caused marked fibrinogenolysis. Infusion of dog brain thromboplastin resulted in the appearance of an anticoagulant which interfered with clot formation on addition of thrombin.

Fibrinogen has certain unique physical and biochemical properties which make a study of its metabolism one of special interest. By definition, fibrinogen is the plasma protein which is clotted in the presence of thrombin. In addition, it is the only protein which irreversibly precipitates from normal plasma at 56° C. These two properties greatly simplify the study of turnover rate and body distribution. The prime physiologic function of fibrinogen is its key position in the blood coagulation mechanism. That it may also play other roles not directly related to hemostasis is suggested by the frequent association of hyperfibrinogenemia with disease (infections, malignancy, etc.).

The plasma level of fibrinogen is controlled by a number of different mechanisms: (1) *Genetic control of synthesis:* Although the genetic evidence from family studies is sparse, the frequency of consanguinity in forebears suggests that congenital afibrinogenemia is due to an abnormal gene. (2) *Increased degradation rate:* The many reports of hypofibrinogenemia associated with fibrinolytic phenomena seem obvious examples. (3) *Unknown mechanisms:* Whether hyperfibrinogenemia (of pregnancy or disease) is associated with increased synthesis or decreased degradation is unknown.

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Supported by grants from the National Health Institutes, United States Public Health Service (H-2254 C), and from the Pittsburgh Health Research and Services Foundation.
Received for publication Jan. 6, 1961.

Turnover rate of fibrinogen has been studied in two ways. In congenital afibrinogenemia,[1, 2] unlabeled fibrinogen has been administered and the half-life estimated at 4 days. In normal humans, dogs, and rabbits isotopically labeled fibrinogen has been used. Estimated half-lives have been as follows: In human beings—5.6 days (S^{35}),[3] (I^{131})[4]; 4.3 days (I^{131}).[5] In dogs—4.2 days (S^{35})[3]; and in rabbits—66 hours (I^{131}),[6] and 69 hours (C^{14}).[6]

This investigation has been undertaken to develop and explore methodology and to study the effects upon fibrinogen survival of certain possibly modifying agents; i.e., fibrinolysins, anticoagulants, and dog brain extract.

Materials and methods

Thrombin. Thrombin, topical (Parke, Davis & Co.), diluted to contain 1,000 National Institutes of Health units per milliliter.

Fibrinolysin. Thrombolysin (courtesy of Dr. N. H. Schimmel, Merck Sharp & Dohme Inc.) administered as noted in Fig. 7.

Preparation of dog fibrinogen. No attempt was made to prepare a highly purified preparation, as it was thought advantageous to have a nonfibrinogen protein component (in order to compare survival times), and as a simple fractionation procedure tended to avoid the denaturation which often accompanies extensive handling. Chilled oxalated or citrated dog plasma was brought to 25 to 33 per cent saturation with ammonium sulfate added dropwise with constant stirring. The mixture was maintained at 0° C. for 30 minutes, centrifuged cold, and the precipitate dissolved in one quarter of the original plasma volume of 0.15 M KPO_4 buffer, pH 7.5. The fraction was dialyzed against repeated changes of the same buffer until free of NH_4 ions by test with Nessler's reagent. Fractionation at lower concentrations of ammonium sulfate resulted in higher fibrinogen concentrations but lower total yield. Among the various fractions used the fibrinogen concentrations varied from 28 to 50 per cent.

Iodination. The fibrinogen fractions were trace-labeled with I^{131} by the tri-iodide method at pH 7.5[7] Free I^{131} was removed by exhaustive dialysis at 4° C. against 0.15 M phosphate buffer, pH 7.5. The fibrinogen was usually passed through a Seitz filter before administration. Administered dose varied from 1.53 to 10.1 mg. protein nitrogen (mean, 7.0 mg.) containing 50 to 60 μc I^{131}. By calculation less than 3 atoms of iodine were bound to a fibrinogen molecule.

Determination of radioactivity. I^{131} gamma activity was measured in a 4 inch lead shielded well scintillation detector with thallium activated sodium iodide crystal. Samples were corrected for background and for decay by comparison with a reference sample. No correction for I^{131} activity withdrawn in the repeated blood sampling was made.

Recipient fibrinogen radioactivity, method H. Five hundred lambda plasma was diluted with 1 ml. 0.85 per cent sodium chloride (pH 7.5) and heated to 56° C. for 5 minutes. After centrifugation the precipitate was washed twice and I^{131} activity determined. Nitrogen was determined subsequently in the same sample by the micro-Kjeldahl method.

Method F. Five hundred lambda plasma was diluted with 1 ml. 0.85 per cent sodium chloride (pH 7.5) and clotted with 0.1 ml. (or more, if necessary) thrombin. The fibrin was wound out on a small glass stirring rod, washed thoroughly, teased off the rod into a vial, and counted. The supernatant was tested for the presence of nonclotted fibrinogen by addition of another 0.1 ml. thrombin. Subsequently the clot was digested and nitrogen content determined.

Method P-S. If coagulation proceeds normally the difference in I^{131} count between plasma and serum might be expected to represent the fibrinogen I^{131}. That this method entails certain inaccuracies due to fibrinogen volume is shown in the *Technical Results* section. One milliliter samples of plasma (anticoagulated with dry Sequestrene-Na_2) and serum (2 or more hours after clotting) were counted. The serum count was also used to determine the survival of nonfibrinogen labeled proteins.

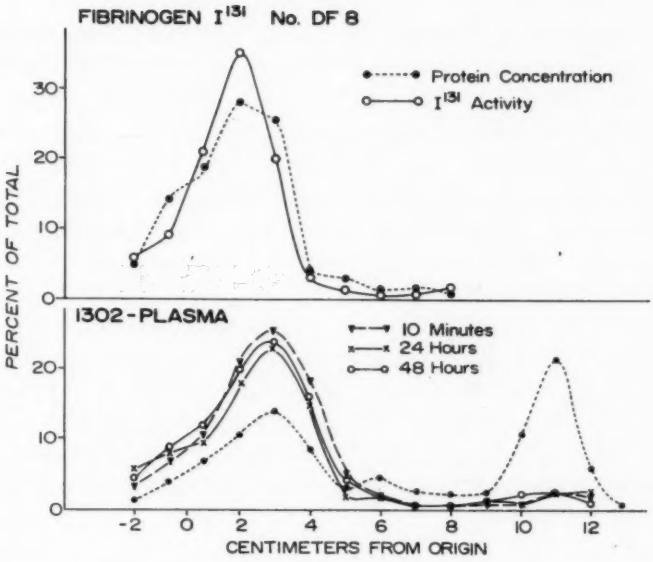

Fig. 1. Paper electrophoresis of dog fibrinogen fraction and dog plasma (see text).

Determination of plasma volume. The Evans blue method was used as described by Warren[8] and Dow.[9]

Prothrombin time and other coagulation tests. In these tests were employed previously described methods.[10]

All dogs received 5 drops Lugol's solution orally for 3 days before an experiment.

Technical results

Iodination of fibrinogen fraction. The top portion of Fig. 1 shows a comparison of protein (bromophenol blue) and I^{131} distribution in a typical fibrinogen preparation after paper electrophoresis. It is evident that I^{131} concentration is proportional to protein concentration.

Distribution of I^{131} in recipient plasma proteins. The possibility that the I^{131} label might shift among the plasma proteins in the recipient plasma was checked in the following experiment. Plasma samples obtained 10 minutes, 24 hours, and 48 hours after fibrinogen I^{131} infusion were each applied to 8 paper strips. After electrophoresis, the papers were cut in 1 cm. sections and each identical lot of 8 rolled up into a tube and counted. The lower portion of Fig. 1 shows the results and should be compared with the upper section, which represents the fraction infused into this dog. No evidence of tag-shift was seen.

Plasma volume determined with fibrinogen fraction I^{131}. If the iodinated protein fraction is promptly and evenly distributed in the plasma it should serve as an accurate method of determining plasma volume. To check this, Evans blue determinations were done on the first group of dogs. Fig. 2 shows the correlation of the two determinations made 10 minutes after the infusion of the dye plus fibrinogen I^{131}. The coefficient of correlation was 0.92. In later experiments only the 10 minute fibrinogen I^{131} value was used for determining the plasma volume.

Comparison of methods of assay of recipient fibrinogen. Fig. 3 shows the mean fibrinogen survival curves in 20 dogs as determined by the three methods. Methods H and F agree well; method P-S shows markedly lower levels of circulating fibrinogen I^{131} toward the end of the experimental period. In fact, some of the late serum samples actually counted higher than the plasma samples. Two explanations are possible: (1) Methods H and F give inaccurately high values due to occlusion or adsorption of nonfibrinogen protein I^{131} onto the precipitate or clot; and (2) method P-S gives an inaccurately low level due to either volume change or splitting off of a soluble iodinated portion of the fibrinogen molecule during coagulation.

The first possibility was tested in the following experiment. Whole serum (previously dialyzed) was iodinated and dialyzed until free of unbound I^{131}. Three or 0.6 ml. samples of this serum were mixed with 10 ml. lots of cold, fresh dog blood (silicone technique) and samples pipetted out into Sequestrene-Na_2 and glass tubes. "Fibrinogen I^{131}," as tested by the three methods at the high and low levels of serum activity, is shown in Table I. At the low level of

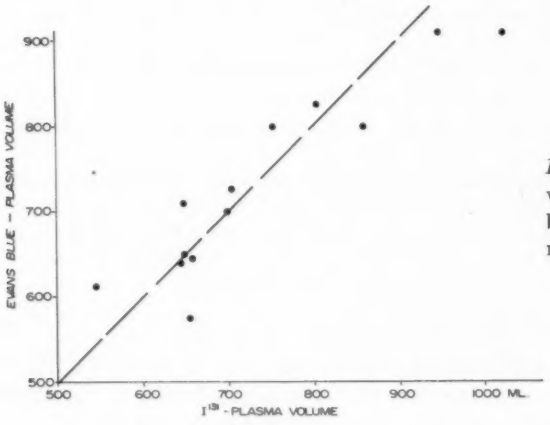

Fig. 2. Comparison of plasma volumes as estimated by Evans blue and fibrinogen I^{131} techniques.

Fig. 2.

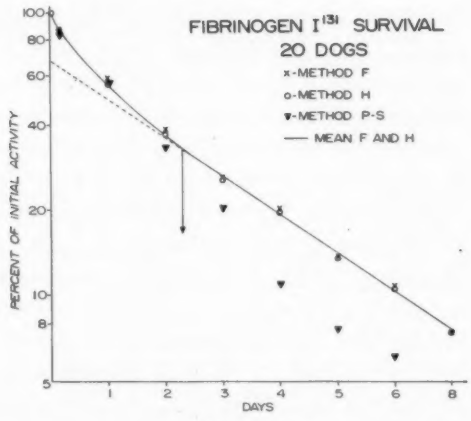

Fig. 3. Comparison of 3 methods of fibrinogen assay.

Fig. 3.

Table I. In vitro tests with serum I^{131}

Material counted	*10 ml. whole dog blood plus:*	
	0.6 ml. serum I^{131}	*3 ml. serum I^{131}*
	(counts/minute/milliliter)	
Whole blood	4,298	21,490
Plasma	5,868	29,830
Method H	33	158
Method F	20	103
Method P-S	−1,176	−3,730

Table II. In vitro tests with fibrinogen I^{131}

Material counted	*10 ml. whole dog blood plus*	
	0.12 ml. I^{131}	*0.8 ml. I^{131}*
	(counts/minute/milliliter × 10^4)	
Whole blood	1.02	6.88
Plasma	1.46	10.91
Method H	0.54	4.02
Method F	0.52	3.93
Method P-S	0.42	3.47

serum activity, which corresponds to that which was found in in vivo experiments, no significant amounts of serum I^{131} were occluded in methods H and F. When the serum radioactivity was increased fivefold, the occlusion was still insignificant, the final counts being less than 3 times background. At both levels of serum radioactivity the serum counted higher than the plasma, suggesting a volume change during clotting.

In a second experiment iodinated fibrinogen fraction (0.8 and 0.12 ml. per 10 ml. blood) was mixed with fresh dog blood and fibrinogen I^{131} assayed by the three methods. Methods H and F were in good agreement, whereas method P-S was low (Table II).

In the entire study some 220 plasma samples from 33 dogs were analyzed by methods H and F and the coefficient of correlation between the results observed in the two methods was 0.996. As will be pointed out later, marked discrepancies between the two methods occurred under certain experimental conditions.

Data presented in ensuing charts employ the mean of methods H and F as the fibrinogen value unless otherwise stated.

In 3 dogs fibrinogen and serum radioactivities were followed for 32 days. During the first 48 hours there was a rapid drop (50 per cent) in both fibrinogen and nonfibrinogen radioactivity. Thereafter, fibrinogen I^{131} disappearance rate slowed, reaching insignificant levels in 8 to 10 days. After the initial equilibration period both fibrinogen I^{131} and nonfibrinogen protein I^{131} disappearance followed exponential curves with half-lives of 2.4 days for fibrinogen and 10 days for the main nonfibrinogen protein component (primarily γ globulin).

In the 20 normal dogs studied the T½ for fibrinogen I^{131} averaged 2.43 days, with a range of 1.8 to 3.05. If the straight line (semi-log paper) portions of the fall-away curves were extended back to zero time, the intercept averaged 66 per cent of the initial value, suggesting a distribution ratio between the intra- and extravascular fibrinogen pools of 1.93.

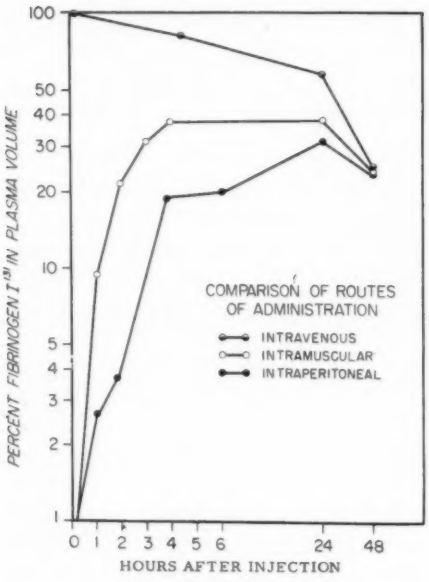

Fig. 4.

Fig. 4. Fibrinogen I^{131} plasma levels following intravenous, intramuscular, and intraperitoneal administration.

Experimental results

Effects of route of administration. Fig. 4 compares the plasma levels of fibrinogen I^{131} obtained after intravenous, intramuscular, and intraperitoneal administration of the same lot of labeled fibrinogen. Evans blue plasma volumes were used for the calculations. By 48 hours the plasma levels were the same.

Equilibration period and distribution in lymph. The rapid fall in intravenously administered fibrinogen I^{131} during the first days and the rather prompt appearance of the labeled fibrinogen in the plasma after intramuscular and intraperitoneal injection suggested that fibrinogen could pass "freely" through the vascular wall. In two dogs the thoracic lymph duct was cannulated,* and the appearance of labeled fibrinogen observed. In one dog a preliminary lymph sample (before heparinization) was obtained for coagulation studies, which are shown in Table III. It was necessary to heparinize both dogs in order to keep the cannulae from being obstructed by clot. Eighteen to 24 hours after operation, when the lymph appeared essentially free of red cells, labeled fibrinogen was administered intravenously, and levels in lymph and plasma followed for 6 hours, as shown in Fig. 5. The results are presented as counts per minute per milliliter. In the lower portion of the chart, per cent of total counts due to

*The authors wish to thank Dr. William Hanna for performing the necessary surgery.

Table III

Protein and coagulation factors in dog plasma and lymph		*Plasma*	*Lymph*
Total protein (Gm./100 ml.)		6.68	5.25
Fibrinogen (methods H and F) % total protein		5.8	2.0
Albumin (paper electrophoresis)		28.8	35.6
α_1 (paper electrophoresis)		4.6	5.8
α_2 (paper electrophoresis)		8.6	8.7
β_1 (paper electrophoresis)		20.7	19.6
β_2 (paper electrophoresis)		14.4	13.8
γ (paper electrophoresis)		22.8	16.4
Clotting time (on whole blood or lymph)	Minutes	6	3
Clot retraction (on whole blood or lymph)		++++	0
Platelet count (on whole blood or lymph)	Per cubic millimeter	330,000	0
Prothrombin time	Seconds	7.0	7.6
Serum prothrombin time	Seconds	18.4	10.2
Plasma-thrombin time	Seconds	25.0	33.0
Assays for (as % plasma value)			
Prothrombin		100	62
VII and X		100	68
V		100	40
VIII		100	75
IX		100	75
Hageman		100	40

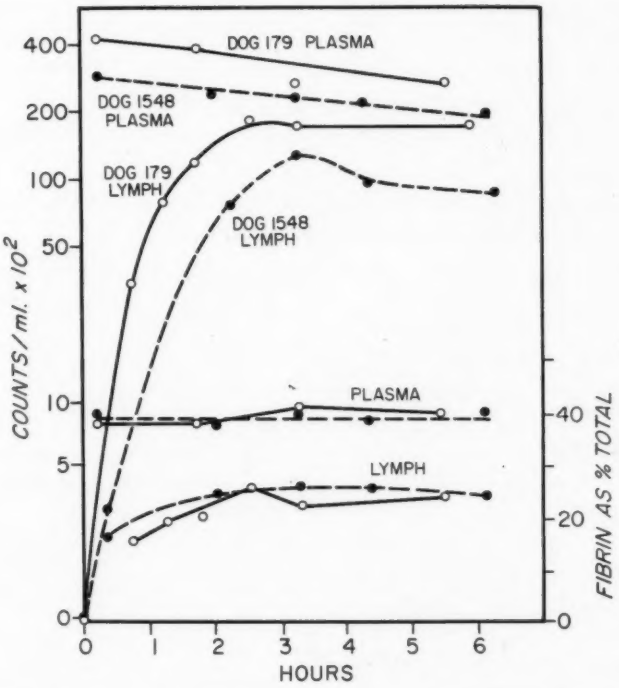

Fig. 5. Appearance of fibrinogen I^{131} in lymph following intravenous infusion. (See text.)

Table IV. Tissue concentration I^{131} (Dog No. 1,302)

	Per cent total injected dose
Lung	0.8
Spleen	0.1
Heart	0.5
Kidney	0.4
Liver	1.4
Gastrointestinal tract	5.6
Lymph nodes	0.1

fibrinogen are shown. In the plasma samples 39 per cent of the total activity was in the fibrinogen fraction (the original fraction also assayed at 39 per cent fibrinogen). Fibrinogen I^{131} appeared in the lymph more slowly than did the labeled nonfibrinogen components and never reached greater than 26 per cent of the labeled lymph protein.

Tissue distribution of I^{131}. One animal was sacrificed 24 hours after infusion of fibrinogen I^{131}. Table IV shows the distribution of radioactivity in selected perfused tissues calculated as per cent of total injected dose.

Effects of anticoagulants on fibrinogen survival. A popular theory concerning blood coagulation states that a continuous process of clot formation and clot lysis occurs in the body at all times. If this theory is true, procedures which deter coagulation might be expected to prolong survival time of fibrinogen.

Three dogs received depot heparin in varying doses so that the coagulation time 10 to 12 hours after injection was 15 minutes or longer. Six dogs received oral Coumadin in dosage to maintain prothrombin time between 2 and 15 per cent. In both groups hemorrhage was a frequent complication, not only from venipuncture sites but also spontaneous, into gastrointestinal tract, mouth, and subcutaneous tissue.

Fig. 6 shows that no significant differences in fibrinogen survival were observed in spite of markedly impaired coagulation.

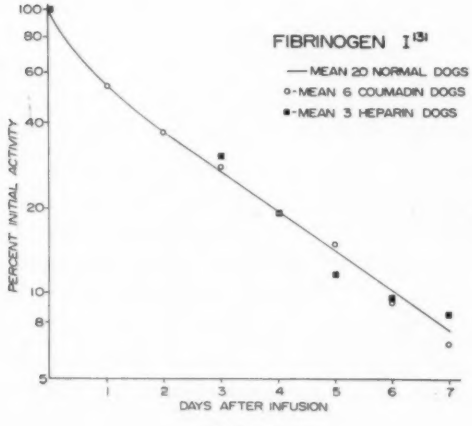

Fig. 6.

Fig. 6. Fibrinogen survival in dogs treated with Coumadin or heparin.

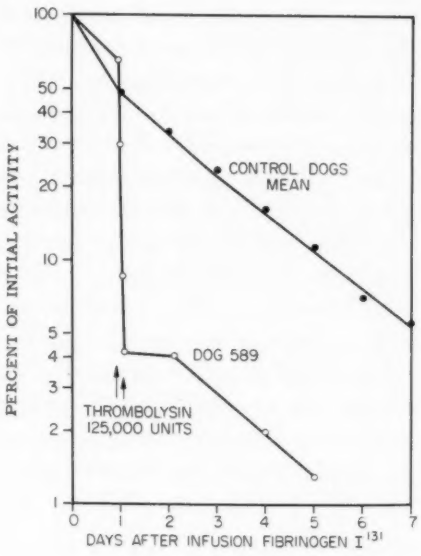

Fig. 7.

Fig. 7. Effects of intravenous infusion of a fibrinolytic agent on fibrinogen survival.

Fig. 8. Effects of intravenous thromboplastin on fibrinogen survival.

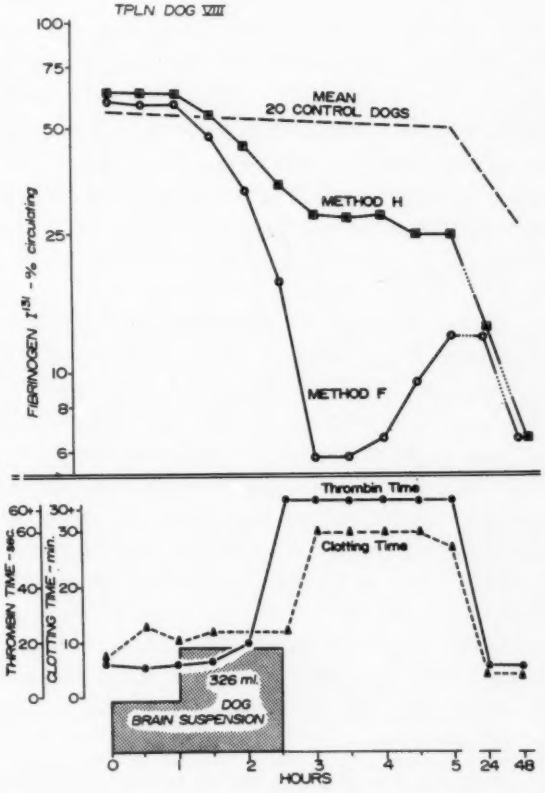

Fig. 8.

Effects of fibrinolysis on fibrinogen survival. Previous experiments had shown that infusion of a fibrinolytic agent, Thrombolysin, prepared by streptokinase activation of human profibrinolysin, was able to cause a marked drop in fibrinogen. It seemed of interest to determine whether or not any of the fibrinogen degradation products might retain the I^{131} label and be reutilized in fibrinogen synthesis. Twenty-four hours after infusion of fibrinogen I^{131} a large dose of Thrombolysin was administered (Fig. 7). During and immediately after the infusion of the fibrinolytic agent a marked fall in total and labeled fibrinogen occurred. Twenty-four hours later the labeled fibrinogen level had changed only slightly, presumably due to reequilibration with extravascular labeled fibrinogen. Thereafter, the rate of fall paralleled that seen in the control dogs. The total (unlabeled) fibrinogen returned to normal levels 24 hours after the initial fall. It should be noted that there was no significant difference between fibrinogen levels as determined by methods H and F although large amounts of thrombin were necessary to clot the fibrinogen in the samples taken after Thrombolysin infusion.

Effects of tissue thromboplastin infusion. Another experimental technique which has been described in the literature as resulting in hypo- or afibrinogenemia is the slow infusion of tissue thromboplastin. We employed a technique similar to that described by Ratnoff and Conley,[11] using a slow infusion of dog brain emulsion. The various coagulation factor changes found will be described in detail in a later communication.[12] A marked discrepancy in fibrinogen levels as measured by the 56° C. and thrombin methods was observed, which suggested that an anticoagulant was present or that the fibrinogen molecule was modified to become resistant to thrombin action (also to spontaneous coagulation) but was still capable of precipitating at 56° C. Fig. 8 demonstrates the results in one experiment.

Discussion

These experiments demonstrate a mean half-life of 2.4 days for iodinated fibrinogen in the dog. This is considerably shorter than that reported by other investigators.[3-6] Technical errors can readily effect the apparent survival time. Incomplete separation of the recipient fibrinogen from contaminating labeled protein might give misleadingly high counts, particularly in the late samples. Decreased survival might result if the fibrinogen molecule was damaged during fractionation or iodination. Another factor which might shorten fibrinogen survival is the presence of a contaminating pyrogen, resulting in a pyrogenic reaction with activation of the fibrinolytic enzyme system. We have no evidence suggesting damage to the fibrinogen molecule or the occurrence of pyrogenic reactions in our experiments. There was no evidence of accumulation of I^{131} in any tissue studied. On addition of thrombin the same amount of fibrin was obtained from the fraction before and after iodination, and 10 minutes after infusion into the dog.

During the first 24 to 48 hours the fibrinogen I^{131} level fell rapidly, suggesting that fibrinogen passed from the circulating pool to an extravascular

pool. Fibrinogen I^{131} appeared in the thoracic lymph by 30 minutes and reached a maximum by 3 to 4 hours. At this time the specific activities (counts per minute per milligram fibrinogen) were approximately the same (24.3 for plasma, 23.9 for lymph). This rate of equilibration of fibrinogen I^{131} between plasma and lymph is somewhat more rapid than that described by Wasserman and Mayerson[13] for albumin I^{131}. These investigators found an equilibrium point at approximately 7 to 8 hours.

Although the plasma and lymph appeared to reach an equilibrium by 4 hours, the rapid phase of fibrinogen disappearance from the plasma continued for at least 24 hours. These observations suggest the possibility of three fibrinogen compartments, one intravascular, one a rapidly exchanging extravascular, and a third, more slowly exchanging extravascular. If the slow phase exponential decay curve, which, presumably, measures rate of fibrinogen metabolism, is extended to the zero line, the mean intercept is at 66 per cent of the initial plasma activity, indicating an extravascular pool size of approximately 50 per cent of the plasma volume.

The presence of iodinated fibrinogen in the lymph demonstrates that this large molecule can pass the vascular "wall" without undergoing coagulation, in spite of the fact that the potential ingredients for clot formation are also present in lymph fluid.

No evidence to substantiate the theory[14] of continuous intravascular fibrin deposition and lysis was obtained. Anticoagulants did not lengthen the survival time of fibrinogen.

Infusion of a fibrinolytic agent caused fibrinogenolysis. In the 24 hour period after the rapid decrease in plasma fibrinogen I^{131} level there was no additional fall. Although it is possible that labeled fibrinogen split products were reused in the formation of new fibrinogen, a more likely explanation is the re-equilibration of intra- and extravascular labeled fibrinogen molecules. Thereafter the plasma fibrinogen I^{131} fell at a rate paralleling that of the control dogs.

Slow infusion of a potent dog brain thromboplastin resulted in prolonged plasma-thrombin and whole blood coagulation times. Additional experiments, to be published elsewhere, showed a variety of coagulation factor changes, the most impressive of which were a profound thrombocytopenia and an inability of the plasma to clot on addition of potent thrombin. The latter finding resulted in the marked discrepancy between fibrinogen as measured by methods H and F (Fig. 8) and could, at least in part, be attributed to the presence of an inhibitor. Ratnoff and Conley[11] and Hartmann and associates[15] found evidence of abnormal thrombin-fibrinogen reaction following infusion of homologous brain extracts.

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Study of fibrinogen and plasminogen concentrations in rabbits during anaphylactic shock

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In order to evaluate the effect of anaphylaxis on the last stages of coagulation, the different clotting parameters of these stages were determined in blood obtained from rabbits before and after induction of anaphylactic shock.

Two cubic centimeters of dog serum, injected into an ear vein on 2 consecutive days, served to sensitize the animals. Sixteen days after the last injection anaphylaxis was provoked with 0.4 to 1.2 cm.³ of dog serum.

Blood obtained from an aortic catheter was processed for its fibrinogen and plasminogen concentration and the plasmin and plasminogen activator activity. Fibrinogen concentrations were also studied in animals pretreated with epsilon aminocaproic acid before injection of the anaphylaxis-provoking dose of dog serum.

It was found that fibrinogen concentrations invariably declined after induction of anaphylaxis. This was not found in the group of animals pretreated with epsilon aminocaproic acid.

Plasminogen concentrations also declined invariably after induction of anaphylaxis. Concomitant with the decline in plasminogen concentration there occurred a substantial increase in plasminogen activator activity. These changes are consistent with the hypothesis that activation of the plasminogen-plasmin system occurs in anaphylaxis in rabbits.

In anaphylactic shock several abnormalities of the coagulation system have been described. Jaques and Waters[11] have shown that the clotting time is prolonged in anaphylaxis in the dog. They have indicated that the marked prolonged clotting times are a result of increase in circulating heparin.

Pesci,[17] and more recently, Quick and associates,[19] have shown that agglutination of platelets and severe thrombocytopenia occur during anaphylaxis. Associated with disintegration of platelets there may be intravascular coagulation.

Plasminogen activation in vitro by antigen-antibody reaction has been incriminated by Ungar and Mist.[23] This activation has not been reproduced by others.[12]

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Supported by United States Public Health Service Grant No. H-534I.
Received for publication Sept. 27, 1960.

Fibrinogen and plasminogen-plasmin system were studied during anaphylaxis in rabbits. The results indicated that the fibrinogen concentrations do fall after induction of anaphylactic shock. Considerable activation of the plasminogen-plasmin system was demonstrated after induction of anaphylactic shock.

Materials and methods

Albino rabbits weighing 2 kilograms were used for all the experiments referred to in this paper. Each of these animals were given, as a sensitizing dose, 2 intravenous injections of 2 ml. of whole dog serum on 2 consecutive days.[20]

Twelve to 16 days following the administration of the sensitizing dose, the animal was subjected to intubation of the aorta under Nembutal anesthesia. An anaphylaxis-provoking dose of 0.4 to 1.2 ml. of dog serum was then given through the cannula in the aorta.

One group of animals was pretreated with epsilon aminocaproic acid (E.A.C.A.)* in a dose of 6 to 8 ml. (0.15 M) per rabbit.

The severity of the ensuing anaphylactic reaction was judged by the extent of the blanching of the ear veins, dyspnea and cyanosis, the increase in small bowel motility, and the distention of the abdominal veins.[4, 20] Whole blood (5 ml.) was withdrawn before and after injection of dog serum. These samples were collected in tubes containing 0.25 ml. of 10 per cent Sequestrene.

The blood was centrifuged for 5 minutes at 2,000 r.p.m. and the plasma separated. Plasma samples were placed on the different fibrin plates (see below), after which the rest of the plasma was quick frozen. Thawing and rewarming of the samples was done at 37° C. in a constant temperature bath. All samples of one rabbit were processed simultaneously on the day of collection for their fibrinogen and plasminogen contents.

Fibrinogen concentrations. These were determined by the method of Kjell-Jacobsson.[10] The plasma is diluted with buffer and clotted with a standard thrombin preparation. The clot is washed and dissolved in a 40 per cent urea solution. The solution is read in a Beckman spectrophotometer at 279, 320, and 360 mμ against a urea blank.

Plasminogen concentrations. These were determined by the casein digestion method. The plasmin and plasminogen of serum or plasma are freed from their inhibitors by being precipitated in the euglobulin fraction at pH 5.2. The euglobulin solution is incubated with standard amounts of streptokinase, dilution of human serum and casein for 30 minutes, and the amount of casein digestion determined by measuring the tyrosine content of the reaction mixture in the Beckman spectrophotometer at 280 mμ. Plasminogen determination by this method yields the same values for both plasma and serum. Therefore, the euglobulin plasminogen values are not dependent on fibrinogen concentration.

FIBRIN PLATES. The plates were prepared according to directions given by Astrup and Mullertz.[3] Fibrinogen is precipitated from bovine oxalated plasma with ammonium sulfate. The fibrinogen fraction is dissolved in barbiturate buffer (pH 7.8) to a final concentration of 200 mg. per cent. Ten cubic centimeters of this solution is pipetted into standard Petri dishes and clotted with 50 u. thrombin.†

HEATED PLATE. The above Astrup plate is heated at 80° C. for 30 minutes.[13]

Plasminogen was obtained from plasma Fraction III‡ by a fractionation method described by Kline.[12A] Ten grams of Fraction III is acidified by addition of 200 cm.³ of 0.05 N sulfuric acid. After stirring and centrifugation, the top layer of lipids is skimmed off. The remainder is brought to pH 11 with 1 N NaOH. After exactly 1 minute the pH of the solution is brought to 5.3 with 1 N HCl. After refrigeration for 3 hours, the pH is again brought to 2. Solution is centrifuged in the cold for 1 hour at 2,500 r.p.m. The gelatinous residue is discarded and the supernatant is brought to pH 8.6 and dialyzed against sodium

*Ortho Research Laboratory, Ortho Pharmaceutical Corporation Raritan, N. J.
†Topical Thrombin, Parke, Davis & Company, Detroit, Mich.
‡Plasma Fraction III obtained through the courtesy of Dr. Gerlough of E. R. Squibb & Sons, New York, N. Y., and Dr. J. N. Answorth of the American Red Cross.

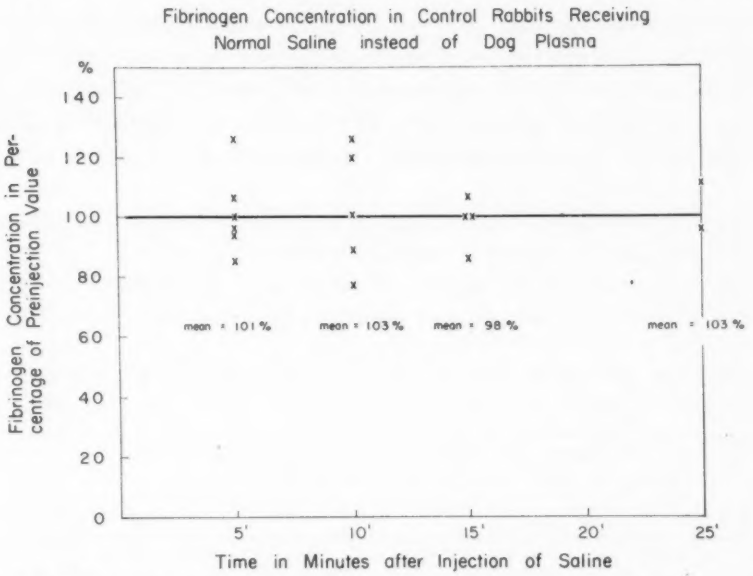

Fig. 1. Fibrinogen concentrations expressed in percentage of preinjection value, in 6 control rabbits receiving saline instead of dog serum.

phosphate buffer (0.025 M, pH 6.0). Dialysate is refrigerated 1 hour and resulting precipitate is separated by centrifugation at 2,500 r.p.m. for 1 hour. Precipitate is dissolved in 25 cm.^{3} of distilled water to which is added one drop of 0.1 N HCl. This plasminogen solution is stored in refrigeration.

PLASMINOGEN REINFORCED PLATE. Of the above standard plasminogen solution, 0.1 cm.^{3} was added to the usual constituents of the Astrup plate.

Epsilon aminocaproic acid was used in a 0.15 M solution. The E.A.C.A. (1 cm.^{3}) was added to the constituents of the plasminogen reinforced plate. These plates are described as epsilon aminocaproic acid–enriched plates (E.A.C.A. plates).

The amount of lysis of the fibrin plates was determined as described by Astrup[3] by measuring the greatest diameter of the lysed area after incubation at 37° C. for 20 hours. Duplicate applications of 30λ were placed on surface of each plate.

Results

Fibrinogen concentrations. Since the initial fibrinogen concentration varies normally from one animal to the other, the concentrations found after injecting the animals were expressed in a percentage of preinjection values.

Six control rabbits received saline 10 to 15 days after the primary sensitizing injections of dog serum. No marked changes were observed after saline injection (Fig. 1).

Upon challenging 12 animals with dog serum, however, and then provoking anaphylactic shock, a decline in the fibrinogen concentration invariably occurred (Fig. 2). The average decrease in fibrinogen value for these 12 animals was found to be 44 per cent.

The group of 12 rabbits pretreated with E.A.C.A. prior to injection of the provoking dose of dog serum showed no significant changes in the fibrinogen concentrations (Fig. 3).

Plasminogen plasmin system. The effect of in vitro combination of dog serum and rabbit blood obtained from previous sensitized animals was studied specifically. This addition did not result in the activation of the plasminogen-plasmin system. The lack of activity was demonstrated by incubating samples of this combined plasma and its euglobulin fractions on all the types of fibrin plates.

Plasminogen concentration. No changes of any significance of the plasminogen values were found in the group of control animals (Fig. 4).

However, plasminogen concentrations as determined by Norman's[16] method invariably declined after the induction of anaphylaxis (Fig. 5). The maximum

Table I. Fibrinogen concentrations in mg. % before and after induction of anaphylatic shock in rabbits

No. rabbit	*Fibrinogen value (mg. %) Time (in minutes) after induction of anaphylaxis*						*Remarks*
	Control	*5*	*15*	*30*	*45*	*60*	
129	363	215					Dead within 8 minutes
132	398	148					Dead within 6 minutes
149	360	205					Dead within 6 minutes
158	335	295	230	260	180	228	No shock
160	200	72	74 c. p.*				Dead within 15 minutes
167	354	212	177	155 c. p. at 20			Dead within 30 minutes
169	322	116	103 c. p.				Dead within 15 minutes
170	497	244	495	475			No shock
2,288	386	320	230	210			No shock, marked anaphylaxis
2,289	442	306	226 c. p.				Dead within 15 minutes
2,296	230	197	141				Dead in 18 minutes
2,297	310	272	252				No shock

*c. p. = Cardiac puncture.

Table II. Fibrinogen concentrations in mg. % before and after induction of anaphylaxis in rabbits pretreated with epilson aminocaproic acid

No. rabbit	*Fibrinogen value (mg. %) Time (in minutes) after induction of anaphylaxis*						*Remarks*
	Control	*5*	*15*	*30*	*45*	*60*	
142	255	275	268 c. p.*				Dead within 15 minutes
143	396	373	366 c. p.				Dead within 15 minutes
145	328	312	392 c. p.				Dead within 15 minutes
146	481	561	425	412			Animal killed with Nembutal
170	315	295	372	420	410		No shock
171	483	398	412 c. p.				Dead within 15 minutes
173	355	335	322				Dead within 20 minutes
175	311	335	335				Dead within 18 minutes
177	300	288 c. p.					Dead within 5 minutes
180	413	398 c. p.					Dead within 5 minutes
181	304	357 c. p.					Dead within 5 minutes
182	290	290	265 c. p.				Dead within 15 minutes

*c. p. = Cardiac puncture.

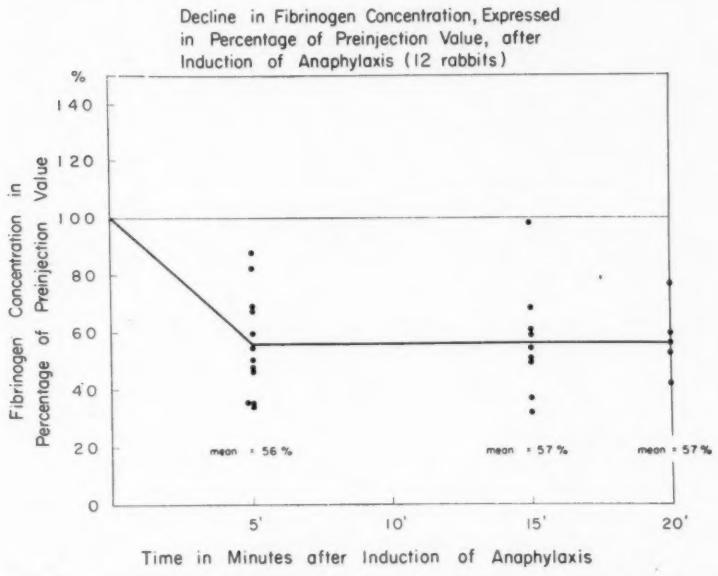

Fig. 2. Decline in fibrinogen concentration, expressed in percentage of preinjection value, after induction of anaphylaxis in 12 rabbits.

decline occurred in the first 5 minutes and decreased to 58 per cent of the original concentration. No activity was noted in the heated (Lassen[13]) plates. Therefore, no free plasmin was found.

Plasminogen activator activity. Concomitant with the plasminogen decrease found after induction of anaphylaxis, a substantial increase in plasminogen activator activity was noted. This increased plasminogen activator activity was determined by the extent of lysis of the plasminogen reinforced fibrin plates

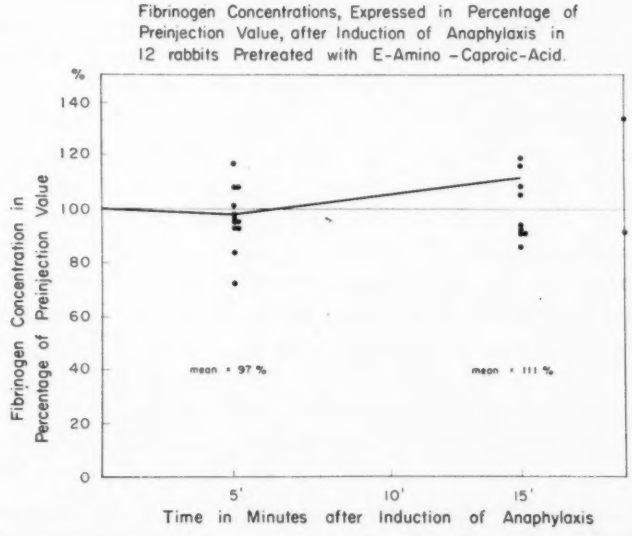

Fig. 3. Fibrinogen concentrations, expressed in percentage of preinjection value, after induction of anaphylaxis in 12 rabbits pretreated with epsilon aminocaproic acid.

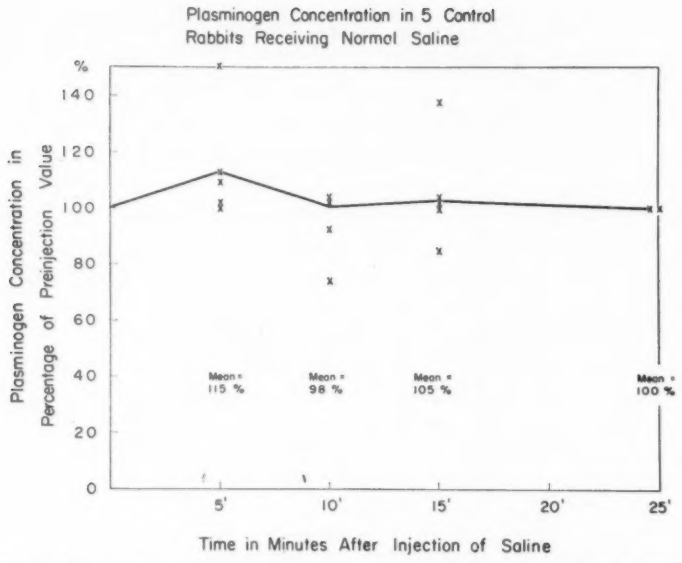

Fig. 4. Plasminogen concentrations, expressed in percentage of preinjection value in 5 control rabbits receiving normal saline.

produced by euglobulin samples. The increase in plasminogen activator activity was found in the 5 minute sample and reached a maximum in the 15 minute sample (Fig. 6). Lysis on the plates increased from an average control level of 40 mm. to a maximum of 160 mm.[2]

Course of anaphylactic reaction. No difference in the symptoms of anaphylactic reaction could be detected between the E.A.C.A.–pretreated rabbits and the unpretreated animals (Tables I and II).

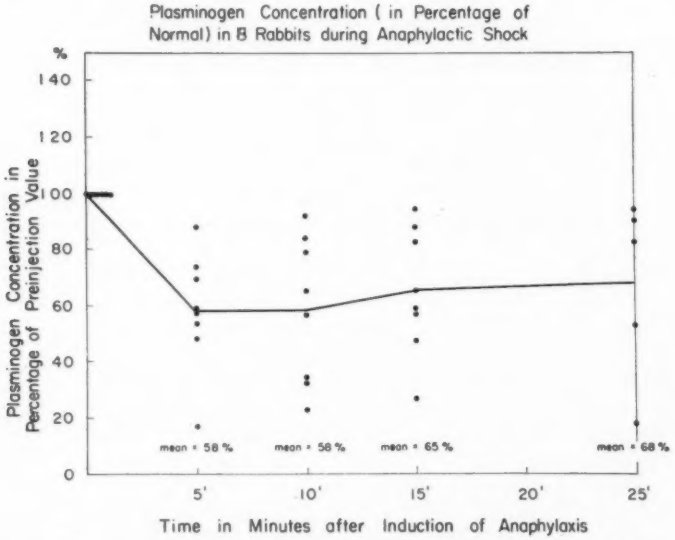

Fig. 5. Plasminogen concentrations, expressed in percentage of preinjection value, in 8 rabbits after induction of anaphylaxis.

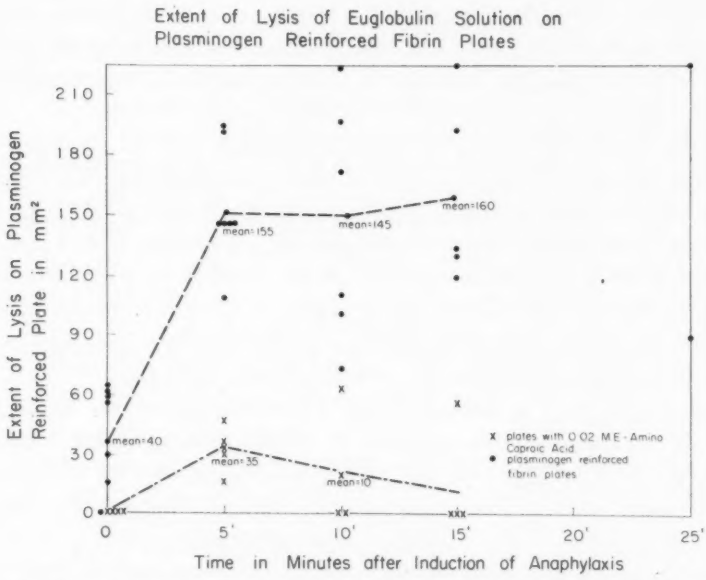

Fig. 6. Increase in plasminogen activator activity, as is evident from the extent of lysis of the plasminogen reinforced fibrin plates, after the incubation of euglobulin samples prepared from the plasma of 8 rabbits. Increase in plasminogen activator activity, as evident from the extent of lysis of plasminogen reinforced fibrin plates, after the incubation of euglobulin samples prepared from the plasma of 8 rabbits. Note here that E.A.C.A. fibrin plates have minimal lysis. This inhibition by E.A.C.A. indicates that plasminogen activator activity was responsible for the observed lysis.

Discussion

In reviews of Doerr[4] and Richet[20] the methodology for producing anaphylactic shock was outlined. The symptoms of anaphylactic shock of the rabbits in the present experiment were those described by these authors. The effect on the rabbits reported here were impressive in that there was a dramatic occurrence of dyspnea, cyanosis, hyperperistalsis with defecation, and vasoconstriction and death occurred in the animals at the time of induction of anaphylaxis.

These animals undergoing anaphylactic shock were studied in order to evaluate the effect of the condition on the fibrinogen and plasminogen-plasmin system.

The first change noted was the decline in fibrinogen concentration. This partial change occurred immediately after induction of the anaphylaxis. The average decline was found to be 44 per cent of the original fibrinogen concentration. This fall in fibrinogen was consistent in the experimental animals and absent in the control animals.

The apparent decrease of the fibrinogen concentration can be explained by several different postulates. These postulates, however, have to take into account the fact that the noted fibrinogen changes were prevented by pretreating the animals with E.A.C.A. which acts as a powerful inhibitor of plasminogen activation. The effect of several activators of plasminogen, like streptokinase,[1] urokinase,[15] and human plasminogen activator[9] is completely inhibited by the

compound E.A.C.A. This, then, supplied correlative indirect evidence suggesting that the fibrinogen decline was the result of the activation of the plasminogen-plasmin system, since the decline in fibrinogen level was prevented by pretreating the animals with E.A.C.A.

Direct evidence for activation of the plasminogen-plasmin system was obtained by observing a decrease in plasminogen concentration and a rise in plasminogen activator activity. The plasminogen decline of 58 per cent from the original value was not present in the control animals. The plasminogen activator activity rose in inverse proportion to the decline in plasminogen.

The interpretation of the above noted findings must include a conclusion that anaphylaxis activates the plasminogen-plasmin system. E.A.C.A. did not protect the animals against signs and symptoms of anaphylaxis.

The lack of lysis on the heated fibrin plates indicates no demonstrable plasmin activity. Plasmin is known to be readily inactivated. Therefore absence of detectable free plasmin activity remains compatible with an activation of the plasminogen plasmin system. Indeed, hyperplasminemia is probably only rarely noted in conditions of active fibrinolysis.[22]

In the past, several investigators have tried to demonstrate plasminogen activation during anaphylaxis. Rocha e Silva,[21] in experiments on anaphylaxis, added protamine to neutralize the heparin released in dogs. Subsequently, however, Downie and Cliffton,[5] showed that protamine itself has marked plasminogen activating properties. Because of this, the mechanism of plasminogen activator which Rocha e Silva observed remains unsolved.

Subsequently, Ungar and Mist[23] demonstrated in an in vitro system that antigen addition to guinea pig blood containing sufficient quantities of antibodies resulted in the activation of plasminogen. Several investigators,[6, 12] however, were unable to confirm these findings. Similarly, the experiments presented here are unable to demonstrate plasminogen activation in vitro.

Rocha e Silva[21] has postulated that the proteolytic mechanism releases histamine, serotonin, and bradykinin. In the present experiments, plasminogen activator activity is inhibited by pretreatment with E.A.C.A., yet anaphylaxis still occurs. Suggestively, proteolysis as represented by the plasminogen-plasmin system cannot be implicated in the causative mechanism of signs and symptoms of anaphylaxis.

The activation of the plasminogen-plasmin system observed after anaphylaxis is in marked contrast to the lack of such activation in the rabbit following the induction of shock from other causes such as adrenalin[24] or endotoxin.[14] This laboratory has been able to confirm the observation that endotoxin shock in rabbits does not activate the plasminogen-plasmin system.

The ability of the rabbit to activate the plasminogen-plasmin system in response to anaphylaxis but not to other types of insult remains a paradox. Each type of shock produced may have a particular end-organ response, i.e., lungs in anaphylaxis, kidneys in endotoxin, and intestines in hemorrhagic shock. Perhaps the plasminogen activator activity of these organs may be sufficiently different to explain the above apparent discrepancies. Further investigation along these lines is in progress.

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The correlation between mouse virulence and the phage type, antibiotic sensitivity, or source of *Staphylococcus aureus*

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Studies were carried out on strains of staphylococci to determine if differences in virulence could be detected which correlated with the phage type, antibiotic sensitivity, or the source of the infecting organism. By the methods employed, no difference in virulence between the strains could be detected.

Because the recent literature contains many reports incriminating *Staphylococcus aureus* of phage type 80/81 as the cause of numerous epidemics of staphylococcal infections throughout the country,[1-4] it has been postulated that this strain is more virulent than other staphylococci.

Amsterdam and Schneierson[5] could find no direct relationships between penicillin susceptibility and virulence of *Staphylococcus aureus*, but they made no reference to the phage type or source of the strain tested.

This study was made to determine if differences in mouse virulence could be detected between strains of *Staphylococcus aureus* which correlated with the phage type, antibiotic sensitivity, or source of the infecting strain.

Materials and methods

All strains of staphylococci used in this study were isolated from clinical sources in the hospital. Sensitivity to the following antibiotics was determined by the disk method, using high concentration disks only: penicillin, tetracycline, dihydrostreptomycin, bacitracin, novobiocin, erythromycin, and chloramphenicol. Phage typing was done according to the procedure of Blair and Carr[6] with the phage being applied with a multiple-typing apparatus designed in this laboratory.

Supported in part by a grant from the Research and Development Command, United States Army Contract No. DA-49-007-MD-959.

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Received for publication Dec. 9, 1960.

Virulence was determined by kidney infection studies and LD_{50} determinations. For the kidney studies, 0.05 ml. of an 18 to 24 hour Trypticase* soy broth culture was injected into a tail vein of white, albino mice which weighed approximately 20 Gm. The mice were checked daily, and deaths, if any, were recorded. Those remaining alive for 7 days were sacrificed, and the kidneys were removed aseptically. The gross appearance of the kidneys, as described by Smith and Dubos,[7] was used to determine whether or not infection was present. Regardless of the appearance of the animals' kidneys, each pair was placed in a Potter-Elvejhem homogenizer. The procedure described by Smith and Dubos[7] was followed through the remaining part of the experiment. Tenfold dilutions of each homogenate were plated and counted. In all cases where the gross appearance of the kidneys seemed normal, no organisms were found on the plates.

For the LD_{50} studies, a similar culture was injected intraperitoneally into mice along with 5 per cent gastric mucin.† The LD_{50} was calculated by the method of Reed and Muench,[8] using 48 hour survival times as the end point.

Table I. Correlation between mouse kidney infectivity and the source of the staphylococcal strain

Source of organism	*Average No. organisms per kidney pair*	*Per cent deaths*
Wounds	3.3×10^7	21
Pneumonia	2.9×10^7	0
Septicemia	2.9×10^7	25
Carriers		
Nose	2.2×10^7	22
Throat	1.6×10^7	27

Table II. Correlation between phage type and mouse kidney infectivity

Phage type	*Average No. organisms per kidney pair*	*Per cent deaths*
80/81	2.4×10^7	13
Others	3.1×10^7	26

Results

Kidney infection studies. Table I shows the correlation between the source of staphylococci and mean numbers of organisms cultured from the kidneys of the mice, and the deaths due to these strains. The data show that the kidneys were infected to approximately the same extent. Death occurred in some of the mice after the injection of staphylococci from 3 of the 5 sources, but no real trend was established.

The results of the correlation between the virulence of phage type 80/81 strains of staphylococci and other phage types are shown in Table II. The data again show that there is no apparent difference between the mouse virulence of 80/81 strains and the other phage types studied.

*Trypticase obtained from Baltimore Biological Laboratory, Md.
†Gastric mucin obtained from The Wilson Laboratories, Chicago, Ill.

Table III. Summary of mouse kidney infectivity studies in relationship to the source, phage type, or antibiotic sensitivity of *Staphylococcus aureus*

Strain No.	*Phage type*	*Strain source*	*No. mice infected*	*No. kidney pairs infected*	*No. mice died*	*No. organisms cultured per kidney pair*	*Antibiotic resistance patterns*
1	44A	Furuncle	10	2	2	1.5×10^7	Sensitive
2	80/81	Wound	10	7	2	6.3×10^7	P, Te, DS, C, E*
3	80/81	Pneumonia	4	3	–	1.7×10^7	P, Te, DS, C, E
4	80/81	Wound	10	8	–	1.5×10^7	P, Te, DS, C, E
5	80/81	Septicemia	6	6	–	2.0×10^7	P, Te, DS, C, E
6	80/81	Carrier (throat)	9	4	5	1.5×10^7	P, Te, DS, C, E
7	80/81	Wound	4	3	1	2.1×10^7	P, Te, DS, C, E
8	80/81	Carrier (throat)	9	7	–	1.6×10^7	P, Te, DS, C, E
9	80/81	Carrier (nose)	10	8	–	2.5×10^7	P, Te
10	42D/44A	Pneumonia	6	6	–	4.1×10^7	Sensitive
11	53/77	Septicemia	9	7	2	1.7×10^7	P, Te, DS
12	6/47	Wound	10	1	4	4.4×10^7	Sensitive
13	42E/75	Wound	10	4	2	2.5×10^7	P, Te, DS, C
14	53/77/83	Septicemia	7	4	3	4.1×10^7	P
15	29/42E/47	Wound	9	4	2	3.4×10^7	Sensitive
16	6/42E/47/70/73	Carrier (nose)	10	6	4	1.9×10^7	Te

*P, penicillin; Te, tetracycline; DS, dihydrostreptomycin; C, chloramphenicol; E, erythromycin.

Table IV. Summary of LD_{50} studies in mice using various strains of staphylococci

Phage type	*Resistance patterns*	*Source*	*No. LD_{50} organisms*
Negative	P, Te, DS, C, E*	Urine	1.8×10^6
Negative	Sensitive	Boil	4.7×10^5
42E/75	P, Te, DS, C	Wound	3.1×10^6
80/81	P, Te, DS, C, E	Autopsy	1.3×10^6
80/81	P, Te, DS, C, E	Wound	2.5×10^6
80/81	P, Te	Carrier (throat)	8.6×10^5
7/29/47/47C/77	Sensitive	Wound	2.9×10^5
6/29/42E/47/54	Sensitive	Wound	4.3×10^5

*P, penicillin; Te, tetracycline; DS, dihydrostreptomycin; C, chloramphenicol; E, erythromycin.

Table III shows a summary of all strains studied. Obviously, no difference in virulence was detected by this method on these strains of staphylococci regardless of the source, phage type, or their sensitivity patterns.

LD_{50} studies. Table IV summarizes the data collected by determining the LD_{50} of 8 strains of staphylococci isolated in this hospital. Again, no significant difference in virulence was detected. Even though there were slight differences in the number of organisms causing an LD_{50}, no consistency is noted according to the source, phage type, or sensitivity pattern.

Invasiveness study. Since the 80/81 strain causes a large percentage of the infection in hospitals,[1-4] it was thought that this strain might possess the ability to invade the host and establish an infection more readily than other strains, thus preventing the strains of less common phage types from establishing a lesion in the kidney.

Five hundredths milliliter of a washed suspension containing an equal number of organisms of 2 different strains of staphylococci was injected into a tail vein of 2 groups consisting of 10 mice each. In the first experiment, one strain was an 80/81 phage type and the other a type 53/77/83. Both strains were isolated from a patient with septicemia. The other experiment was conducted using strains of the same phage patterns but from different sources. After 7 days, kidney counts were made as described earlier in this paper. A plate which contained at least 15 colonies was chosen at random from each kidney dilution. Each colony was subjected to phage-typing procedures. The results show that both strains were present to the same degree on the plates, indicating that the kidneys were infected with both organisms to approximately the same extent. (Table IV.)

Table V. Phage typing results of staphylococcal strains isolated from infected mouse kidneys after simultaneous intravenous injections of 2 different phage types

Group No.	*Average No. colonies/plate*	*Average No. phage type 80/81*	*Average No. phage type 53/77/83*
I	18	9	9
II	15	6	9

Discussion

Studying the difference in virulence between coagulase-positive and coagulase-negative strains of staphylococci by intravenous injections into mice, Smith and Dubos[7] found that the coagulase-positive strains survived within the kidneys for 7 days, but the coagulase-negative strains were greatly diminished. Although they did not imply that degrees of virulence could be detected in this way, it was thought that this method might serve to clarify the controversy about whether or not there are degrees of virulence between the strains of staphylococci commonly found in hospital patients and personnel.

Kidney infection experiments carried out in this laboratory failed to show any difference in mouse virulence between strains of staphylococci that correlated with their source, phage type, or antibiotic sensitivity patterns. This was also true with the LD_{50} studies.

The finding that 2 strains of staphylococci of different phage types infected the kidneys of mice to the same extent agrees with the work of Gorrill.[9] Studies were not carried out, as his were, to determine the number of foci of infection because we were interested only in discovering if the phage type 80/81 strains had such invasive characteristics that they could invade the host and outgrow the other strains, thus eliminating them from the host. It was found that this was not the case; both strains were recovered in approximately the same numbers from each pair of infected kidneys. Since this is the case, it would seem that there should be more mixed-wound infections found, especially those that have been attributed to environmental conditions. It should be mentioned that this experimental approach to the problem may be in favor of the invading organism, since both organisms were introduced directly into the blood stream. Thus they escaped a most important host barrier, the skin. Work on this problem is being continued in our laboratory.

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Relation between quantitative nasal cultures and dissemination of staphylococci

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Quantitative nasal cultures, skin cultures, and air samples using slit-samplers have been made on 250 hospitalized patients. Only 4 per cent of skin cultures from patients who were not nasal carriers of staphylococci and 5 per cent of skin cultures from patients who were nasal carriers of less than 100,000 colonies per culture contained coagulase-positive staphylococci. However, 44 per cent of cultures of the skin of patients who were nasal carriers of more than 100,000 colonies contained staphylococci. Aerial dissemination of staphylococci during activity was also greater in patients who were nasal carriers of large numbers of bacteria. Over 20 colonies per cubic foot of air could be recovered from 35 per cent of air samples around patients who were carriers of over 100,000 colonies per culture but from only 8 per cent of air samples from patients who were either noncarriers or carriers of less than 100,000 colonies. Carriers of small numbers of staphylococci appear to be no greater hazard in the dissemination of staphylococci than are patients who are not nasal carriers.

In several studies[1, 2] a correlation between nasal carriers of staphylococci and staphylococcal infections has been described. Patients who were nasal carriers acquired postoperative infections and other types of staphylococcal disease more frequently than patients who were not nasal carriers. Also, a higher incidence of staphylococcal infections has been reported in the families of infants who were nasal carriers and who disseminated staphylococci in large numbers than has been reported in the families of infants who were also nasal carriers but who disseminated staphylococci poorly.[3]

The increased dissemination of staphylococci by infants who were nasal carriers was associated with concomitant viral infections. Therefore, it was postulated that viral infections might increase the number of staphylococci available for dissemination, increase mucous production and greater dissemination, or alter surface tensions and viscosity of the nasopharynx so that the same number of staphylococci might be disseminated more readily.

From the University of Louisville Department of Medicine, Louisville, Ky.
Supported by Grant No. E-2561 from the National Institute of Allergy and Infectious Diseases, Bethesda, Md.
Received for publication Dec. 16, 1960.
*Markle Scholar in Medical Science.

In previous studies of quantitative nasal cultures for staphylococci among hospitalized patients, it was found that staphylococci could be isolated more frequently but not in larger numbers from the clothing of nasal carriers of large numbers of staphylococci as compared to either carriers of smaller numbers of organisms or to noncarriers.[4] Cultures of clothing were made by sweeping open Petri dishes over the patients' gowns, a relatively insensitive method for quantitating bacterial contamination. These studies have been continued using more accurate methods for measuring aerial dissemination; again, staphylococci could usually be isolated more frequently from the environment of heavy nasal carriers and, in addition, larger numbers of staphylococci were present more frequently in positive air samples collected around heavy carriers than in air samples collected around light carriers or noncarriers.

Methods

The methods of collecting and enumerating quantitative nasal cultures and phage typing of staphylococci were those described previously.[5] All staphylococci were coagulase-positive.

Air samples were collected near the beds of patients on the wards of the Louisville General Hospital using slit-samplers[6] at the rate of 1 cubic foot of air per minute. Only samples from patients without overt staphylococcal infections were included. Since we were attempting to observe conditions as generally present on the ward, no attempts were made to either increase or decrease activity during the period of sampling, but one sample from each patient was made while the patient was resting quietly in bed and one sampling from each patient was made while the sheets were shaken lightly for 15 seconds during the collection period.

Skin cultures were made by swabbing an area 3 by 1 inches on the forearm with a cotton swab moistened with heart infusion broth. The swab was then placed in a culture tube with 3 ml. of heart infusion broth and shaken for 5 minutes in a Kahn shaker. The broth was then enumerated for staphylococci as reported for nasal cultures.

Results

Coagulase-positive staphylococci were present in 4 per cent of skin cultures from patients who were not nasal carriers of staphylococci and in 5 per cent of skin cultures from patients who were carriers of less than 100,000 colonies per swab (Table I). However, staphylococci could be isolated from 44 per cent of

Table I. Correlation between quantity of staphylococci in the nose and the frequency of staphylococci on the skin

No. nasal staphylococci	*No. patients*	*Positive skin cultures*		
		No.	*%*	
0	139	6	4	
10^1-10^2	2	0	0	5
10^2-10^3	13	1	8	
10^3-10^4	10	1	10	
10^4-10^5	16	0	0	
10^5-10^6	18	7	39	44
$> 10^6$	23	11	48	
Total	221	26	12	

Table II. Correlation between numbers of staphylococci in the nose and in the air while patients were resting

No. nasal staphylococci	*No. patients*	*Positive air sample*		*> 5 colonies/ cubic foot*		*> 10 colonies/ cubic foot*	
		No.	*%*	*No.*	*%*	*No.*	*%*
0	112	13	12	5	4	2	2
10^1-10^2	1	1	100	0	0	0	0
10^2-10^3	9	0	0	0	0	0	0
10^3-10^4	10	4	40	2	20	0	0
10^4-10^5	16	2	13	2	13	2	13
10^5-10^6	16	4	25	3	19	2	13
$> 10^6$	23	5	22	3	13	2	9
Total	187	29	16	15	8	8	4

skin cultures from patients who were nasal carriers of more than 100,000 colonies per swab. Seventy-five per cent of staphylococci isolated from the skin of nasal carriers were the same phage type as those isolated from the nose.

Air samples were collected near 187 patients while they were resting quietly in bed (Table II). There were only slight differences in the frequency with which staphylococci could be isolated from the air around patients who were not nasal carriers than from that around either light or heavy nasal carriers. However, large numbers of staphylococci were present more frequently in air samples from patients who were nasal carriers of over 100,000 colonies per swab than in air samples from either noncarriers or carriers of less than 100,000 colonies. Seventy per cent of staphylococci isolated from air samples around nasal carriers were the same phage type as those isolated from the nose.

When the patients' sheets were shaken lightly for 15 seconds during the collection period, staphylococci were present in air samples approximately twice as frequently as when the air samples were collected with the patients resting quietly (Table III). Furthermore, staphylococci were isolated both more frequently and in larger numbers from air samples around nasal carriers of more

Table III. Correlation between numbers of staphylococci in the nose and in the air during activity

No. nasal staphylococci	*No. patients*	*Positive air sample*		*> 10 colonies/ cubic foot*		*> 20 colonies/ cubic foot*	
		No.	*%*	*No.*	*%*	*No.*	*%*
0	120	25	21	18	15	10	8
10^1-10^2	4	1	25	0	0	0	0
10^2-10^3	12	3	25	2	17	2	17
10^3-10^4	10	4	40	2	20	1	10
10^4-10^5	11	5	45	2	18	0	0
10^5-10^6	19	11	58	8	42	6	32
$> 10^6$	23	12	52	11	48	9	39
Total	199	61	31	43	22	28	14

than 100,000 colonies per swab than from air samples around either noncarriers or carriers of less than 100,000 organisms per swab. Thus, more than 20 staphylococcal colonies per cubic foot of air were present in 36 per cent of air samples around nasal carriers of more than 100,000 colonies, but only 8 per cent of air samples around either nasal carriers of less than 100,000 colonies or noncarriers contained this many staphylococci.

Twenty-eight strains of staphylococci from samples of air around nasal carriers were the same phage type as staphylococci isolated from the nose. The remaining 8 strains from air samples around carriers and the 25 staphylococci in the air around noncarriers were presumably derived from sources other than the nose of the patient.

Discussion

The role of the many possible sources of staphylococci on the occurrence of staphylococcal infections is not completely established. The largest reservoir of staphylococci, however, appears to be in the nose of patients and personnel, and a number of investigators believe that this is the major source of infections.[7]

Among patients who are nasal carriers there is a wide range of the number of staphylococci which can be recovered from the nose. In this study the number of staphylococci isolated varied from less than 100 to more than one million colonies per culture. Staphylococci could be recovered infrequently from the skin of nasal carriers of small numbers of these organisms but could be recovered from the skin of almost half of the nasal carriers of more than 100,000 staphylococci per swab. Also, air samples around patients who were heavy nasal carriers contained large numbers of staphylococci more frequently than air samples collected around light carriers or noncarriers.

The incidence of staphylococcal infections depends not only on the availability of staphylococci but also on the resistance of the patient to infection. However, other factors being equal, staphylococcal infections might be expected to be more frequent in an environment in which large numbers of staphylococci are present. If the patient population contained a large proportion of heavy nasal carriers of staphylococci, greater aerial dissemination of these organisms would be expected.

It remains to be proved, however, that nasal carriers of large numbers of staphylococci are a greater hazard to the carrier or to other patients in initiating infections than are nasal carriers of smaller numbers of staphylococci.

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Protein fractionation. III. Chromatographic characterization of human erythrocyte cold agglutinins

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Sera of patients with cold agglutinins have been examined chromatographically on columns of diethylaminoethyl cellulose and of hydroxylapatite. With both adsorbents, agglutinating activity was eluted in a single region of the chromatogram which, because of previous characterization of cold agglutinins in other laboratories, precisely locates the 19S γ-globulins in the chromatogram. Ultracentrifugal characterization of chromatographic fractions shows correlation of cold agglutinin activity with the appearance of 19S γ-globulin in the effluent. Analysis of chromatographic fractions by ultracentrifugation reveals multiple components, many of which are not resolvable as separate ultracentrifugal entities in intact serum. One may conclude that they have been separated on the chromatographic column.

In recent years investigations in a number of laboratories have been concerned with the nature and properties of human erythrocyte cold agglutinins, antibody-like molecules arising by what appears to be an autoimmunization process. Gordon[1] prepared cold hemagglutinins by adsorption and elution from erythrocyte stroma and then characterized his purified material as γ-globulin with a sedimentation constant of 18S. Weber[2] also studied cold agglutinins from the sera of 5 patients and found them to have high sedimentation rates (18S). In characterizing the agglutinins of patients with acquired hemolytic anemia, Fudenberg and Kunkel[3] again point out that cold agglutinins are 19S proteins, and while they noted some variation in electrophoretic mobility, the majority were fast γ-globulins. In contrast, the warm antibody associated with this condition in 3 other sera was 7S protein. Christenson and associates,[4] by cellulose column electrophoresis, also characterized cold hemagglutinins as fast (γ_1-)

From the J. K. and Susie L. Wadley Research Institute and Blood Bank, and the Graduate Research Institute of Baylor University, Dallas, Texas.
Presented in part at the One-hundred-thirty-sixth National Meeting, American Chemical Society, Atlantic City, September, 1959.
Supported by a grant from the National Institutes of Health.
Received for publication Jan. 9, 1961.

γ-globulin. With this general agreement concerning the physicochemical nature of the cold hemagglutinins, it seemed that several purposes might be served by chromatographic study of them. First, there is obvious value in adding chromatographic characterization of cold agglutinins to previously described electrophoretic and ultracentrifugal characterization. Second, because of the sensitivity of hemagglutination tests, it was hoped that the cold agglutinins would serve as a marker for the 19S γ-globulins, permitting detection at concentrations considerably below those feasible by ultracentrifugation. This in itself is important in giving increased information about protein chromatography, a process receiving ever wider application to problems of protein purification, and which was recently reviewed by Peterson and Sober.[5] While most of the studies in this report were performed with diethylaminoethyl-cellulose (DEAE) as an adsorbent, a few experiments were also conducted with the hydroxylapatite (calcium phosphate) of Tiselius and colleagues.[6]

Materials and methods

Pertinent patient history. The sera of 2 patients with human erythrocyte cold agglutinins but rather dissimilar clinical histories have been examined in this study. Patient R. K. was a congenitally deaf 35-year-old white man who, for about 3 years prior to being seen in this clinic, had noticed hematuria and facial discoloration after exposure to cold. A diagnosis of cold paroxysmal hemoglobinuria with cold agglutinins has been suggested. Patient A. N. was a 46-year-old white man first seen in the Wadley Clinic in December, 1956, for a severe hemolytic anemia. His case had elsewhere been diagnosed as one of Hodgkin's disease. He underwent splenectomy in January, 1957, and examination of the spleen at that time revealed no sign of malignant lymphoma. He has continued to be anemic and to have demonstrable cold agglutinins in the serum. Neither of these patients had received blood transfusions for a number of months prior to the time of study.

Preparation and evaluation of chromatograms. The methods used in these studies are for the most part the same as those previously described[7, 8] and will be repeated here only briefly. Most of the chromatograms were developed on DEAE-cellulose packed to a height of 25 to 30 cm. in columns of 19 to 22 mm. inside diameter. Development was by gradient elution, with each buffer being added from a 500 ml. dropping funnel to a 500 ml. Wolff bottle which served as mixing chamber. All connections were airtight. Identical buffers used with each column for protein elution were of the following composition: constant glycine, 0.015 M; in the following sequence the molarity of sodium phosphate and pH are given, respectively: I, no phosphate, 9.0; II, 0.02, 8.5; III, 0.04, 8.0; IV, 0.06, 7.5; V, 0.08, 7.0; VI, 0.10, 6.5; VII, 0.30, 6.0; VIII, 0.50, 5.5; IX, 1.00, 4.5. Chromatograms obtained using hydroxylapatite as adsorbent were with columns 9 to 12 mm. inside diameter packed to a height of about 10 cm. Gradient elution was again utilized, but with a mixing chamber of only 250 ml. capacity. Buffers of the following composition were used for development: constant NaCl, 0.2 M; in the following sequence the molarity of sodium phosphate and pH are given, respectively: I, 0.005, 6.8; II, 0.06, 7.0; III, 0.13, 7.0; IV, 0.20, 7.0; V, 0.30, 6.0; VI, 0.50, 5.0.[9] Several chromatograms were developed with a precipitate prepared from serum R. K. by adding to it at 4° C. an equal volume of saturated ammonium sulfate. All of the chromatography was performed at 4° C.

Absorbency measurements were made with a Beckman DU spectrophotometer at 280 mμ. Electrophoresis and ultracentrifugation were performed on samples prepared by combining fractions, as indicated by capital letters, in the chromatograms. They were dialyzed against distilled water, freeze-dried, and redissolved in a minimal volume of 0.9 per cent NaCl. Electrophoresis was performed on paper with Spinco Model R, with the Analytrol used for densitometric analysis. The majority of the patterns were obtained in Veronal buffer, pH

8.6, μ equaling 0.075; some determinations, however, were performed in the TRIS buffer of Aronsson and Gronwall.[10] With the TRIS buffer, patterns were also obtained with the Model R and S and S 2043A gl paper at a constant voltage of 87 volts, 25° C., 17 hours. Ultracentrifugation was conducted in the Spinco Model E with analytical rotor A. The tabulated S values are uncorrected for the variations in protein concentration. To determine the proportions of the various sedimenting components, the Schlieren patterns were projected by means of a photographic enlarger and traced onto graph paper; then planimetry was performed with the Analytrol. Protein was determined by the Folin method as described by Lowry and colleagues.[11] Serologic studies were by tube tests with saline suspended group O, Rh-negative erythrocytes or red cells suspended in saline after papain treatment.[12]

Results

Sera R. K. and A. N. were selected because of their high titers of cold agglutinins. Titration data, along with electrophoretic and ultracentrifugal characterization of the proteins of these sera, are summarized in Table I. The findings for Serum A. N. are essentially normal, but for Serum R. K. there is a significant rise in the serum macroglobulins (normally about 4 per cent) and an alteration of the alpha$_2$ and beta globulin components.

Figs. 1 and 2 show whole serum chromatograms for Sera A. N. and R. K. on DEAE-cellulose. It is to be noted that for both sera the cold agglutinin activity appears in a single chromatographic region and that peak activity is found following addition of buffer VII. This portion of the chromatogram is complex electrophoretically, exhibiting not only small amounts of gamma globulin, consistent with the presence of cold agglutinins, but also other globulins, and, in some instances, small amounts of albumin. In Table II are presented the electrophoretic and ultracentrifugal characterizations of the combined fractions from the chromatogram of Fig. 1. These electrophoretic patterns, obtained in TRIS buffer, are somewhat better resolved than those usually observed for chromatographic fractions performed in Veronal. The various electrophoretic groups from the column emerge in the same order as previously has been noted.[8, 13, 14] Gamma globulins emerge first, then beta globulins, albumin, and finally, alpha globulins.

Table I. Serum protein characterization

Determination	*Serum R. K.*		*Serum A. N.*	
Serologic titer				
37°	1:8		1:1	
25°	1:4		1:2	
4°	1:8,000		1:4,000	
Electrophoresis	% Protein		% Protein	
Albumin	57.9		54.5	
Alpha$_1$ globulin	5.6		5.7	
Alpha$_2$ globulin	5.6		8.0	
Beta globulin	15.0		12.5	
Gamma globulin	15.9		19.3	
Ultracentrifugation	S_{20}	% Protein	S_{20}	% Protein
	15.5	9.4	15.6	2.5
	5.8	20.6	6.1	13.3
	3.4	70.0	3.7	84.2

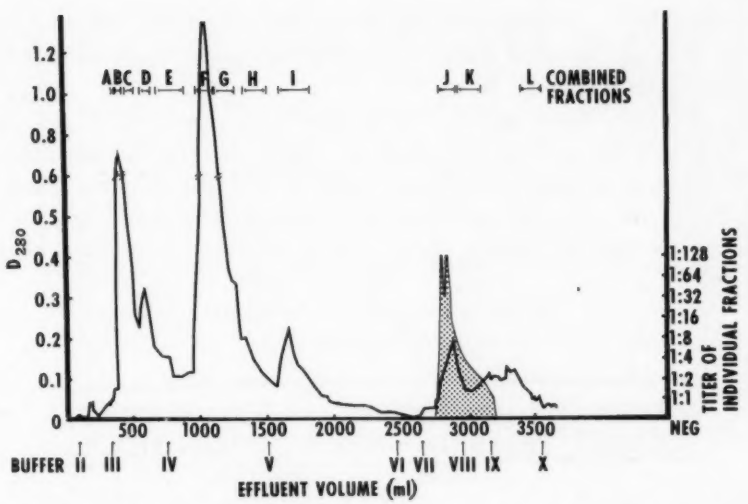


Fig. 1. DEAE-cellulose chromatogram of 10 ml. Serum A. N. Flow rate: somewhat variable but averaging 20 ml. per hour; recovery: 87 per cent.

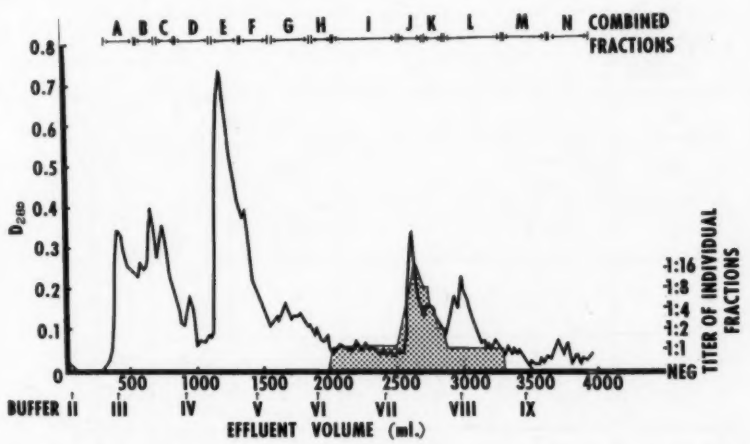


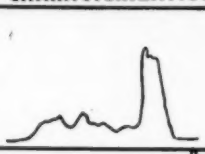
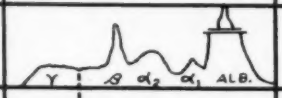
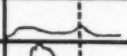
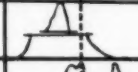
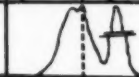
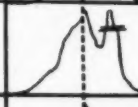
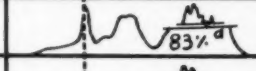
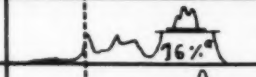
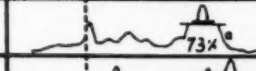
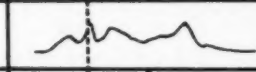
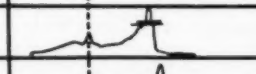
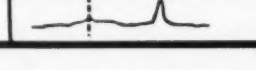
Fig. 2. DEAE-cellulose chromatogram of 8.5 ml. Serum R. K. Flow rate: 22 ml. per hour; recovery: 98 per cent.

The hump seen at the point of application is indicative of protein denaturation occurring as a result of the concentration process. To facilitate comparison of mobilities of peaks in the fractions with those in whole serum, electrophoretic patterns, which were obtained simultaneously, are placed with the origins along a common vertical line. That there are components with mobilities different from those of the whole serum protein groups is evident. An additional common finding is the variation in mobility of albumin in fractions F through I and, similarly, in that of γ-globulin in fractions B through E. For fractions with complex patterns, the percentage of the protein (albumin) in the main peak has been indicated. The ultracentrifugal characterization of the combined fractions is seen to be complex, with sedimenting components not apparent in the original serum being observed. Of particular significance to this report is the

occurrence of the most rapidly sedimenting component in fraction J, the fraction which also contains the peak cold agglutinin activity. A similar correlation was observed for Serum R. K.

When the ammonium sulfate precipitated protein from Serum R. K. was placed on a DEAE column, the chromatogram of Fig. 3 was obtained. The albumin, which accounted for 25 per cent of the precipitate, emerged in its usual place (*G* and *H*); compared to the corresponding peak in Fig. 2 (*E* and *F*), this peak is reduced in size. In Fig. 3, *A* and *B* are gamma$_2$, *C* is gamma$_1$,

Table II. Characterization of combined fractions of DEAE-cellulose chromatogram of Serum A. N.

COMBINED FRACTION	ELECTROPHORETIC CHARACTERIZATION	ULTRACENTRIFUGAL CHARACTERIZATION		
		PROTEIN CONC. (mg./ml.)	S	%
SERUM An		10	15.6 6.1 3.7	2.5 13.3 84.2
NORMAL SERUM				
A		1.4	7.4 5.1	26.7 73.3
B		14.8	9.0 7.3 5.2	4.7 23.5 71.9
C		13.8	7.2 5.2	12.4 87.6
D		9.7	7.0 6.3 4.0	4.1 32.8 63.1
E		7.8	7.3 4.7 3.8	6.1 67.6 26.4
F		5.8	9.5 b 3.8	1.1 98.9
G		3.1	11.7 b 3.7	0.6 99.4
H		7.3	2.8	100
I		14.7	4.1 2.8	17.1 82.9
J		8.1	13.3 5.1 3.3	7.6 53.8 38.7
K		2.6	6.0 3.9	33.3 66.7
L		0.76	—	—

[a] Per cent of protein in main albumin peak.
[b] Detected at 10 × indicated concentration.

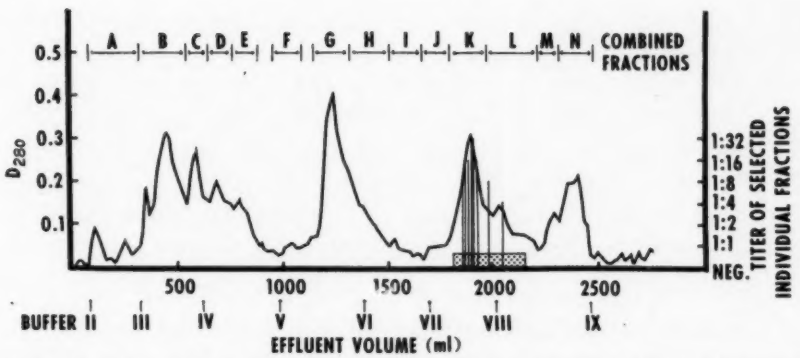


Fig. 3. DEAE-cellulose chromatogram of the protein precipitated by half-saturated ammonium sulfate from 7.5 ml. Serum R. K. Flow rate: 15 ml. per hour; recovery: 82 per cent. The shaded area represents cold agglutinin activity; the vertical lines rising from it give titers of individual fractions.

D is gamma$_1$ with the beginning of the beta peak, and *E* and *F* are both beta globulin. Cold agglutinin activity again is seen to be confined to a single chromatographic region, appearing after introduction of buffer VII. While ultracentrifugation was performed on each combined fraction, it is sufficient for present purposes to point out that combined fraction K, in which peak cold agglutinin activity appeared, contained the typical serum macroglobulin (35 per cent 18.1S) and lower molecular weight globulin (50 per cent 6.3S) but, in addition, a 26S component, comprising the remaining 15 per cent. Electrophoretically, this fraction was 74 per cent beta and 26 per cent gamma globulin.

Several chromatograms were developed on hydroxylapatite; the one for whole Serum A. N. is shown in Fig. 4. Some unadsorbed material containing a small

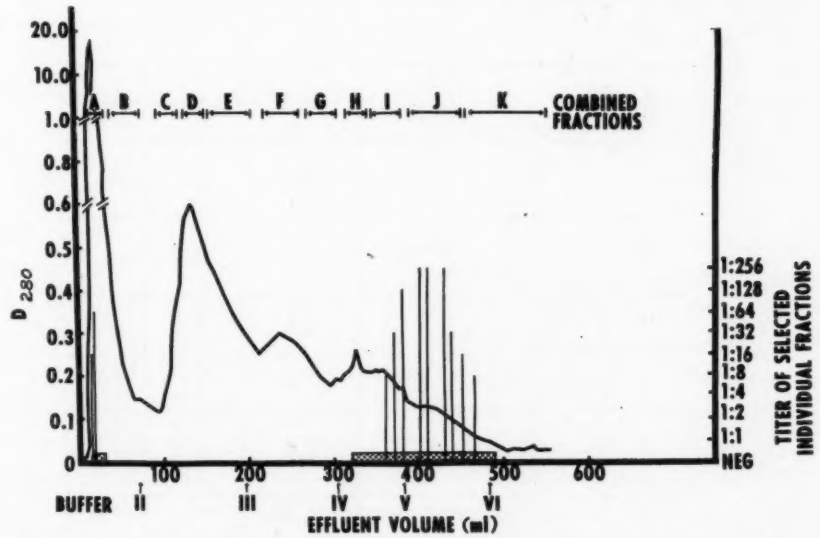
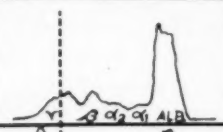
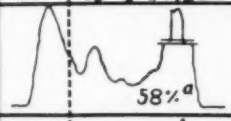
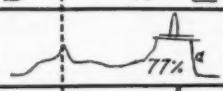
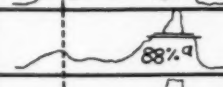
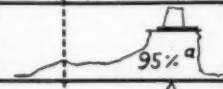
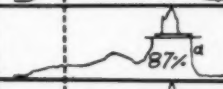
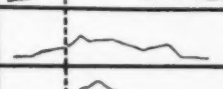
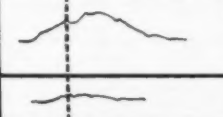
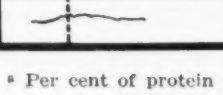


Fig. 4. Hydroxylapatite chromatogram of 5 ml. Serum A. N. Flow rate: 5 ml. per hour. Recovery: 71 per cent. The shaded area represents cold agglutinin activity; the vertical lines rising from it give titers of individual fractions.

amount of cold agglutinin appeared early in the chromatogram, but the bulk of the activity is eluted following addition of buffers IV and V. The same observation was made when the precipitate from Serum R. K. was similarly chromatographed. In Table III are recorded the electrophoretic and ultracentrifugal characterizations of the combined fractions from the hydroxylapatite column (Fig. 4). Following the unadsorbed material, a number of fractions (C through G) are principally albumin. The difference between the 100 per cent observed ultracentrifugally and the somewhat smaller percentages found electro-

Table III. Characterization of combined fractions of hydroxylapatite chromatogram of Serum A. N.

COMBINED FRACTION	ELECTROPHORETIC CHARACTERIZATION	ULTRACENTRIFUGAL CHARACTERIZATION		
		PROTEIN CONC (mg./ml.)	S	%
SERUM An.		10	15.6 6.1 3.7	2.5 13.3 84.2
A		17.3	14.8 5.8 3.6	1.9 21.2 77.0
B		4.4	5.7 3.9	5.2 94.8
C		4.7	3.7	100
D		10.1	3.8	100
E		13.6	3.4	100
F		6.8	4.1	100
G		3.6	3.9	100
H		2.1	18.4 4.8	12.9 87.1
I		2.0	17.3 5.6	18.6 81.4
J		1.6	16.1 6.2 4.3	18.5 63.2 18.3
K		0.57	—	—

[a] Per cent of protein in main albumin peak.

phoretically may be accounted for in part by the tendency of albumin to tail on paper. The remaining fractions are primarily globulins, and contain cold agglutinin, again in association with appearance of macroglobulins.

Specific cold agglutinin activity was calculated for individual chromatographic fractions by determination of the ratio of titer to protein concentration determined by optical density measurement. Increases in specific activity, over that for the column charge, of 16 and 35 fold were observed for peak fractions of Serum A. N. from the DEAE and hydroxylapatite chromatograms, respectively. For Serum R. K. only a twofold increase was observed for the whole serum chromatogram, while activity was increased 7 times when the precipitate from this serum was chromatographed. With papain-treated red cells the titers with Serum R. K. or fractions derived from it were increased by about 4 tubes. The combined fractions of each chromatogram were examined for cold agglutinin activity; despite the increased concentration, none was observed in any additional region of the chromatogram. When papain-treated cells were used, the leading and tailing edges of the cold agglutinin band were somewhat lengthened, however.

Discussion

The chromatography of cold agglutinin containing sera on 2 quite different types of adsorbents—DEAE-cellulose and hydroxylapatite—has in each instance yielded active material in a single region of the chromatogram. This homogeneity is in contradistinction to the highly heterogeneous distribution of anti-A and anti-D reported previously from this laboratory[7, 8] and in articles by Abelson and Rawson.[15, 16] The pathologic process leading to formation of cold agglutinins also stands in contrast to the process of blood group antibody production by what must be considered normal physiologic mechanisms. In the DEAE chromatograms the cold agglutinins appear after introduction of buffer VII, even when the protein milieu is altered, as it is in the chromatogram of the globulin precipitate from Serum R. K. (Fig. 3). Buffer of the same composition has consistently produced a peak activity for saline agglutinating anti-A and anti-B,[8] from which one may infer that this activity is associated with high molecular weight γ-globulin.

Studies from a number of sources (loc. cit.) indicate that cold agglutinins are 19S gamma globulins. As a result, one may assign the 19S gamma globulins to the chromatographic region for each adsorbent characterized by appearance of cold agglutinin activity. Confirmation is found in the presence of rapidly sedimenting components in fractions containing cold agglutinins. With the sedimentation constants clustering around 7 for the gamma globulins of the early chromatographic fractions (combined fractions A through C, Table II), one may conclude that they have been separated on DEAE columns from the late appearing 19S gamma globulins. This chromatographic separation of 7S and 19S gamma globulins has also been recently shown by Fahey and Horbett[17] and by Lospalluto and associates.[18]

When intact serum is subjected to ultracentrifugation, it is customary to describe the 3 sedimenting components that are observed as 4S, 7S, and 19S proteins. It should be recognized, however, that each of these is composed of protein mixtures that simply are not resolved under the conditions of centrifugation. The ultracentrifugal data of Table II clearly show sedimenting components that are not observed during centrifugation of intact serum and point to the conclusion that they have been resolved on the column. Because sedimentation values are uncorrected, small differences cannot be weighed too heavily, and reported S values are lower than the true sedimentation constants. However, when several sedimenting components are indicated for a single chromatographic fraction, they represent definitely resolved peaks. The complexity of these data is reminiscent of those presented by Wallenius and associates[19] in their ultracentrifugal characterization of serum fractions obtained by preparative electrophoresis. Even such a minor component as the 12S protein reported by these workers is in evidence as a minor contaminant of the albumin in fractions F and G (Table II). Furthermore, a 26S protein corresponding to their 28S gamma globulin was detected in combined fraction K of the serum R. K. precipitate (Fig. 3). A more detailed analysis of ultracentrifugal characterization of DEAE chromatographic fractions for a variety of sera will be presented elsewhere.

The ultracentrifugal data from the hydroxylapatite column (Table III) are considerably simpler than from DEAE columns. Of interest are fractions C through G, which are homogeneous ultracentrifugally. Electrophoretically, the protein is primarily albumin, but with quantities varying from 65 to greater than 95 per cent in the main peak. This variation reflects in part the well recognized tendency of albumin to tail on paper. It may be noted that the percentage of the protein appearing in the tail decreases as the protein concentration increases.

The interest of Dr. J. M. Hill and Ellen Loeb and their cooperation in making available patients for this study are gratefully acknowledged.

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Regulation of arachidonic acid metabolism by a pancreatic factor

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Previous work has suggested an endocrine control of lipid metabolism by the pancreatic alpha cells. A study of the effect of alpha cells on the individual plasma fatty acids by gas-liquid chromatography was undertaken. The plasma fatty acid spectra of depancreatized and pancreatic duct-ligated dogs were compared before and during feeding of potent pancreatic enzymes. Depancreatized dogs maintained with insulin and choline showed a highly significant depression of the relative proportion of arachidonic acid. The total plasma fatty acid level fell concomittantly with arachidonic acid, but the relative proportions of the other fatty acids remained unchanged. Feeding potent pancreatic enzyme preparations elevated the total plasma fatty acids to normal or above, but the arachidonic acid ratio remained depressed. The relative proportions of the other individual fatty acids were unchanged. The "arachidonic acid factor" was absent from such pancreatic preparations as whole gland concentrate and purified lipase.

Results similar to the above were obtained in pancreatic duct-ligated dogs receiving choline. This eliminates the pancreatic alpha cells as the source of the "arachidonic acid factor," and places it in the external secretion. The inability of exogenous enzymes to restore the arachidonic acid proportion to normal indicates continued absence of an endogenous source of this fatty acid.

This study demonstrates the presence of a factor in canine external pancreatic secretion that influences the metabolism of arachidonic acid.

Previous work from this laboratory has suggested an endocrine control of lipid metabolism by the pancreatic alpha cells.[1, 2] Therefore, a study of the effect of the alpha cells on the individual plasma fatty acids was undertaken. This approach was made possible by the introduction of gas-liquid chromatography, which permits qualitative and quantitative analysis of individual plasma fatty acids using mieroquantities of material.

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Supported by Research Grant No. H-2715 from the National Institutes of Health, United States Public Health Service.
Received for publication Jan. 11, 1961.

This study compares the plasma fatty acid spectra of depancreatized and pancreatic duct–ligated dogs. The depancreatized animals were maintained with insulin. Both groups received pancreatic enzymes and choline chloride. It was reasoned this experimental design would demonstrate any effect or effects the pancreatic alpha cells might exert on individual plasma fatty acids, since alpha cells were present in the duct-ligated animals but absent in the depancreatized group.

Materials and methods

Healthy, male mongrel dogs were kept in the kennel for at least 1 week prior to use. They were fed a constant diet of 1 can of horse meat and 1 of dry meal daily. A minimum of 3 control experiments was performed on each animal. Following an overnight fast, blood was drawn from a limb vein; heparinized syringes were used for determination of the plasma fatty acid spectrum by gas-liquid chromatography. The total fatty acid level was also determined. After base line studies were established, 4 animals were depancreatized. Pancreatic duct ligations were performed on a second group of 4 dogs.

Pancreatectomies were performed on Dogs Nos. 1, 2, 3, and 4 with utmost care to insure complete removal of all pancreatic tissue. The animals were kept in metabolism cages for 1 week, and received 2 ml. of Combiotic (penicillin-streptomycin*), 15 to 20 units of NPH insulin, the preoperative food ration, and 500 mg. choline chloride daily. The antibiotic was discontinued after the first week, but the remainder of the routine was maintained until the animals were sacrificed. Sutures were removed in 14 days. The dogs were kept acetone-free, but no attempt was made to achieve aglycosuria. Approximately 2 months later, the above regimen was supplemented with a daily oral dose of 5 Gm. of purified pancreatic lipase† and 2 tablets of whole pancreas concentrate (Viokase‡). Dog No. 1 received 15 Gm. of purified pancreatic lipase and 3 tablets of Viokase until the ninety-seventh postoperative day, and was then reduced to the above dosage. Dog No. 2 expired on the forty-eighth postoperative day and never received enzymes. Dogs Nos. 1, 3, and 4 were maintained until the end of the experiment. Postmortem examination was performed on all animals.

Pancreatic duct ligations were performed on Dogs Nos. 5, 6, 7, and 8. Two ducts were ligated and transected in each animal. Great care was taken to ensure complete separation of the pancreas from the duodenum throughout its entire length. None of these animals developed diabetes mellitus. They were given 2 ml. of Combiotic daily during the first week, and sutures were removed in 14 days. Each dog received 500 mg. choline chloride and the same preoperative diet. Approximately 2 months after surgery, the diet was supplemented with 5 Gm. of purified pancreatic lipase and 2 tablets of whole gland concentrate (Viokase). Dogs Nos. 5 and 6 were sacrificed on the sixty-eighth postoperative day, while Dogs Nos. 7 and 8 were maintained until the ninetieth day. Postmortem examination was performed on all animals.

The plasma fatty acids were prepared for gas-liquid chromatography in the following manner. Heparinized blood was centrifuged, then the plasma was removed and rapidly pipetted into a 4:1 solution of methylal-methanol.[3] The flask was held under a hot water tap until the boiling point was reached, cooled to room temperature, and filtered through Whatman No. 1 filter paper. The precipitate was washed with an additional 5 ml. methylal-methanol solution, and the paper squeezed with a clean glass rod. The filtrate was concentrated to approximately 2 ml. under nitrogen in a 55° C. water bath. The lipids were extracted with three 25 ml. portions of boiling petroleum ether. The petroleum ether was evaporated under nitrogen. Two milliliters of ether and 1 ml. of ethereal diazomethane

*Pfizer Laboratories, New York, N. Y.
†Assay—700 lipase units. Obtained from the Southern California Gland Company, Los Angeles, Calif.
‡Viobin Corp., Monticello, Ill.

Table I. Effect of pancreatectomy on the plasma arachidonic acid level expressed as the per cent of the total fatty acids (mean ± S. E.)

		Postoperative			
		No enzymes			
	Control	*Period 1**	*Period 2†*	*Enzymes*	*P values*
Dog No. 1					
Days		7	51	170	
Total fatty acids (mg. %)	126	157	109	295	
Arachidonic acid (%)	14.6 ± 1.1 (3)‡	13.8 ± 4.8 (2)	2.6 ± 1.3 (3)	4.1 ± 0.8 (13)	<.001
Dog No. 2					
Days		13	48	Died	
Total fatty acids (mg. %)	162	148	102		
Arachidonic acid (%)	12.9 ± 1.0 (4)	13.1 ± 0.7 (2)	3.8 ± 0.8 (4)		< .02 > .01
Dog No. 3					
Days		14	70	150	
Total fatty acids (mg. %)	203	172	117	337	
Arachidonic acid (%)	8.4 ± 0.9 (4)	7.9 ± 1.1 (4)	2.4 ± 0.7 (7)	1.9 ± 1.2 (4)	<.001
Dog No. 4					
Days		27	73	150	
Total fatty acids (mg. %)	180	170	92	333	
Arachidonic acid (%)	7.5 ± 0.3 (4)	7.7 ± 0.6 (7)	1.3 ± 1.2 (3)	1.4 ± 0.9 (5)	<.001

*Before fall of arachidonic acid.
†After fall of arachidonic acid.
‡Number of determinations are expressed in parentheses.

(2.7 per cent) were added. This methylated the unesterified fatty acids which were present.[4] The excess ether and diazomethane were evaporated. The lipids were transmethylated by the addition of 5 ml. of methanol and 0.6 ml. of 0.5 N sodium methoxide.[5] The solution was boiled for 2 minutes, cooled to room temperature, and 10 ml. of saturated potassium chloride was added. The methyl esters were extracted with four 10 ml. portions of petroleum ether, and stored under nitrogen at 5° C. until chromatographed.

A Beckman GC-2 gas chromatography apparatus equipped with a thermal conductivity cell was used. Column conditions were as follows: 18 feet, one-fourth inch O.D. stainless steel, packed with 42 to 60 mesh fire-brick coated with Resoflex*; 240° C. temperature; flow rate 140 ml. per minute of helium; filament current 320 Ma; chart speed one-half inch per minute. Identification of the fatty acids was accomplished by comparing the retention times with those of a known mixture of fatty acid esters. The area of each acid was calculated by the triangulation technique[6, 7] and expressed as per cent of the total fatty acid spectrum of the plasma.

*Cross-linked polyester of adipic acid and diethylene glycol succinate. Obtained from Cambridge Industries, Cambridge, Mass.

A separate 1 ml. aliquot of plasma was used for determination of the total plasma fatty acids. The method of Bauer and Hirsch[8] was modified by methylating the unesterified fatty acids prior to colorimetric determination.[4]

Results

Table I shows the effect of pancreatectomy on the plasma arachidonic acid. The values are expressed as per cent (mean ± S. E.), and are calculated on the basis of the entire plasma fatty acid spectrum. Only arachidonic acid is shown, since there were no significant changes in any of the other fatty acids. The fatty acid spectrum extended from caproic to arachidonic acid. Fig. 1 is a representative control chromatogram showing the plasma fatty acid spectrum.

The postoperative data are divided into 2 periods during which enzymes were withheld and fed. The enzyme-free period in turn is divided into the immediate postoperative period, during which the plasma arachidonic acid level remained normal (Period 1), and the interval during which it fell (Period 2). Following pancreatectomy, arachidonic acid remained normal for 1 to 2 weeks, and then fell. Period 1 persisted for 27 days in Dog No. 4. Pancreatic enzyme feeding was delayed for 48 to 73 days in order to observe the effect of prolonged enzyme deprivation on the plasma fatty acid spectrum. The total plasma fatty acid level fell concomittantly with the relative proportion of arachidonic acid during Period 2. The relative proportions of

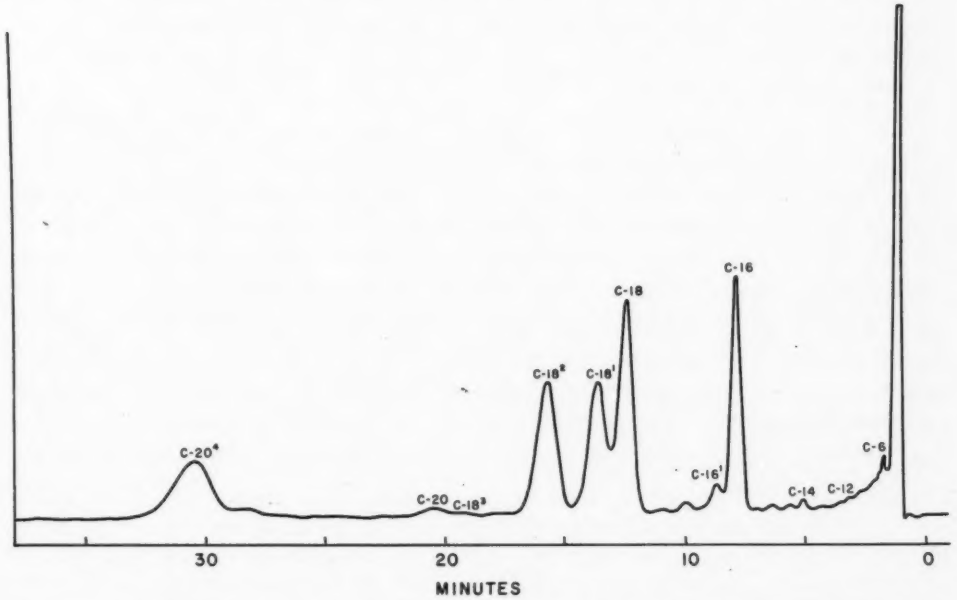

Fig. 1. A representative gas-liquid chromatogram showing separation of fatty acid methyl esters prepared from normal dog plasma. *C* refers to the number of carbon atoms, and the superscript, to the number of double bonds in the molecule. *C-20⁴* identifies arachidonic acid (20 carbons and 4 double bonds). The other major components are: *C-16*, palmitate; *C-16¹*, palmitoleate; *C-18*, stearate; *C-18¹*, oleate; *C-18²*, linoleate; and *C-18³*, linolenate.

the other individual fatty acids did not show any significant change. When pancreatic enzymes were fed, the total plasma fatty acid level returned to normal or above, but the arachidonic acid ratio remained depressed. The other individual plasma fatty acid ratios were unchanged.

The data were submitted to statistical analysis by the P factor and found to be highly significant. It must be pointed out that P was calculated on the basis of all postoperative determinations, including those of Period 1, before the fall of arachidonic acid occurred. If the probability were calculated exclusive of this period, P would be even more significant. The P factor was not as highly significant in Dog No. 2 ($.02 > P > .01$), because this animal expired before the period of enzyme feeding. If the animal had survived, P would have been more significant. Postmortem examination revealed absence of all pancreatic tissue.

Table II shows the effect of pancreatic duct ligation on the plasma arachidonic acid. The values are expressed as per cent (mean ± S. E.).

Table II. Effect of pancreatic duct ligation on the plasma arachidonic acid level expressed as the per cent of the total fatty acids (mean ± S. E.)

		Postoperative			
		No enzymes			
	Control	*Period 1**	*Period 2†*	*Enzymes*	*P values*
Dog No. 5					
Days		6	42	68	
Total fatty acids (mg. %)	141	291	37	107	
Arachidonic acid (%)	8.9 ± 0.5 (7)‡	4.3 (1)	Trace (1)	0 (4)	<.001
Dog No. 6					
Days		6	47	68	
Total fatty acids (mg. %)	156	193	81	113	
Arachidonic acid (%)	9.4 ± 2.0 (8)	5.8 (1)	2.5 ± 2.4 (2)	1.2 ± 1.2	<.001
Dog No. 7					
Days		14	69	90	
Total fatty acids (mg. %)	178	125	80	156	
Arachidonic acid (%)	7.9 ± 0.5 (5)	6.1 ± 0.3 (2)	1.9 ± 1.1 (4)	Trace (3)	<.001
Dog No. 8					
Days		14	69	90	
Total fatty acids (mg. %)	171	48	63	110	
Arachidonic acid (%)	10.6 ± 0.7 (5)	7.3 ± 1.0 (2)	1.7 ± 1.3 (4)	Trace (3)	<.001

*Before fall of arachidonic acid.
†After fall of arachidonic acid.
‡Number of determinations are expressed in parentheses.

Only arachidonic acid is shown, since there were no significant changes in any of the other fatty acids. The results are similar to those obtained in the depancreatized group. The arachidonic acid ratio remained normal for 1 to 2 weeks following duct ligation and then fell, as did the total plasma fatty acid level. Administration of pancreatic enzymes resulted in return of the total plasma fatty acid level to normal or above, but the arachidonic acid proportion remained depressed. When submitted to statistical analysis by the P factor, the results were highly significant in all the animals ($P < .001$). P was calculated on the basis of all postoperative determinations, including those of Period 1.

Postmortem examination on the sixty-eighth postoperative day of Dogs Nos. 5 and 6 revealed atrophy of the pancreatic acinar tissue. The pancreatic remnant was 2 cm. long and one-half cm. across. On histologic examination, a few acini were found. The experiment was terminated in Dogs Nos. 7 and 8 on the ninetieth postoperative day, but the animals were not sacrificed until the two hundred and ninety-ninth day in order that recanalization might occur if duct ligation had been incomplete. A few pancreatic acini were found in the remnant of Dog No. 7 which was 1½ by 1 cm., indicating that canalization probably occurred by the two hundred ninety-ninth day. However, the size of the remnant showed that canalization did not occur until a very late date (the ninetieth day after the experiment was completed) as the pancreas would not have atrophied to such an extreme degree. The fact that a few acini were found in Dogs Nos. 5 and 6 on histologic examination does not mean that duct ligation was incomplete. Sufficient time had not elapsed for complete atrophy of all acinar tissue to occur.

Discussion

This study shows that the external pancreatic secretion of dogs contains a factor that regulates the plasma level of arachidonic acid. It appears to be specific for arachidonic acid, and cannot be supplied by feeding such potent pancreatic enzymes as whole gland concentrate or purified pancreatic lipase. It is probably destroyed during processing of these preparations.

The factor regulates the relative proportion, rather than the absolute level, of arachidonic acid of the total plasma fatty acids. This suggests control of a mechanism affecting the relationship of arachidonic acid and another fatty acid or other acids. One possibility is the conversion of linoleic to arachidonic acid. A number of investigators have demonstrated this conversion in vivo.[9-12]

Removal of the factor did not manifest itself immediately. The relative proportion of arachidonic acid did not fall for approximately 1 to 2 weeks following removal of the external pancreatic secretion by pancreatectomy or pancreatic duct ligation. The total plasma fatty acid level also fell at this time, but the relative proportions of the other fatty acids did not change consistently or significantly. It appears that this period of time is required for the absence of the factor to effect the arachidonic acid ratio. Prolonged withholding of pancreatic enzymes resulted in a further fall of

the arachidonic acid proportion, and the total plasma fatty acid level. When pancreatic enzymes were fed, the total plasma fatty acid level rose to normal or above. In some instances the elevation above normal was twofold. However, the arachidonic acid proportion remained depressed even though its absolute level rose. Increase of the absolute arachidonic acid level is explained by the greater degree of lipid absorption caused by the pancreatic enzymes supplemented in the diet. However, this did not restore the normal ratio of arachidonic acid to the total plasma fatty acids. Impaired absorption cannot be the explanation for depression of the arachidonic acid ratio. If it were, one would expect the relative proportion as well as the absolute plasma level to be restored to the preoperative value when pancreatic enzymes were fed. The inability of exogenous enzymes to correct this indicates continued absence of an endogenous source of this fatty acid; e.g., conversion of linoleic to arachidonic acid.

The fact that similar results were obtained in depancreatized and duct ligated animals eliminates the pancreatic alpha cells as the source of the "arachidonic acid factor" and places it in the external pancreatic secretion. Depancreatized animals receiving insulin lack "alpha cell secretion." Duct-ligated animals with the pancreas in situ have alpha cells. Absence of the external pancreatic secretion is the only condition common to both groups. Therefore, the arachidonic acid factor must be in the external secretion.

Arachidonic acid has several features which distinguish it from other fatty acids. It does not exist in the plant kingdom. Consequently, all arachidonic acid in an animal must be acquired from animal food or be synthesized in vivo. Since tissues of herbivorous animals contain arachidonic acid, synthesis is the only source in these species. It is not present in depot fat but has been shown to be a constituent of mitochrondia,[13] which is the probable explanation for previous reports[14] of its presence in depot fat. The arachidonic acid content of tissues has been reported to remain constant during fat deprivation.[15] It is one of the 3 essential fatty acids, and is more effective than linoleic or linolenic acids which constitute the other members of this group in correcting the symptoms of essential fatty acid deficiency and promoting growth.[16-18] Recent investigations have shown that the unsaturated fatty acids lower plasma cholesterol.[19-22] Arachidonic acid is the most effective in man. Linoleic acid has slight activity, while linolenic acid is ineffective.[23] The primary function of archidonic acid appears to be of a more basic nature, thus differing from that of the other fatty acids, which are mainly a source of energy. Therefore, it is not surprising to observe the existence of a factor influencing the metabolism of this fatty acid specifically. This factor may be of etiologic significance in disease.

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Studies of the pharmacology of o,p′DDD in man

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Blood, tissue, urine, and stool samples from 18 patients being given massive doses of o,p′DDD for the treatment of cancer were studied for the presence of o,p′DDD and a metabolic chromogen. From these studies, it appears that o,p′DDD is from 35 to 40 per cent absorbed from the gastrointestinal tract, appears in the blood stream, and is stored primarily in fat-containing tissues.

Approximately 25 per cent of the drug absorbed each day appears in the urine in a metabolized form. A smaller and variable percentage, also in the metabolized form, appears in the stool via the biliary tract.

The differences of responsiveness of these patients to treatment could not be explained by these studies.

During the past 2 years, the compound 2,2 (4 chlorophenyl, 2 chlorophenyl) 1,1 dichloroethane (o,p′DDD) has been used by us to treat metastatic adenocarcinoma of the adrenal cortex. Not only has this therapy resulted in regression of tumor metastases and remission of steroid side effects, but it has demonstrated suppression of normal adrenal function as well.[1-3]

Because of the availability of a sensitive spectrophotometric method for determining o,p′DDD concentrations in tissues, blood level studies could be performed at various times in most of these patients. To a lesser extent, studies were done on absorption and excretion of the compound as well as on determinations of concentrations in the body tissues as these became available from biopsy and postmortem material.

The results to be reported are admittedly incomplete in terms of a finished pharmacologic study. It is believed justifiable to report the results we have obtained because of the potential medical importance of this compound and because reports of such studies in humans following ingestion of massive doses of this and related insecticides are few.

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Received for publication Jan. 11, 1961.

Studies in experimental mammals have shown many similarities in the pharmacology of DDT, p,p'DDD, and, from the few studies available, of o,p'DDD as well.[4-6, 9] Among these are absorption, primarily in fat and fat-containing tissues, and detoxification and urinary excretion, primarily in the form of the acetic acid derivative DDA (dichlorodiphenyl acetic acid). It was anticipated that this would be true of o,p'DDD in the human as well.

Spectrophotometric determinations of o,p'DDD were performed in a Beckman B spectrophotometer by Cueto and associates'[6] modification of the Schechter-Haller method.[8] O,p'DDD from the lot being given to the patients was used as a standard* and readings were made at 595 mμ.

No interfering chromogens for o,p'DDD or the metabolite discussed below were found when control samples of blood, stool, urine, fat, and adrenal were processed by the method. Reproducibility of duplicate samples from patients consistently showed less than 5 per cent variation and, similarly, known amounts of o,p'DDD added to blood, fat, and adrenal from control patients were better than 95 per cent recoverable.

When the urine of patients receiving o,p'DDD was extracted and subjected to nitration, no o,p'DDD was identified but rather another chromogen appeared that had maximum absorption at 500 mμ. Since this chromogen appeared only in patients who were receiving or had received o,p'DDD it was believed that the chromogen represented products of the metabolism of o,p'DDD. This chromogen also was found in the blood and tissues where, by means of an alkaline wash, it could be extracted from o,p'DDD for separate determinations.†

Ofner and Calvary[5] state that the alkaline soluble metabolites of DDT, primarily dichlorodiphenyl acetic acid, are converted by the intensive nitration of the Schechter-Haller method to tetranitrodichlorobenzophenone. Since purified samples of possible metabolites of o,p'DDD were not available, the assumption was made that the alkaline soluble 'metabolite' found in our patients was, like that of DDT, converted by nitration to the end product tetranitrodichlorobenzophenone. Accordingly, for purposes of quantitation, 2,4 dichlorobenzophenone was used as a standard and nitrated with the tissue extracts. Since the original metabolite or metabolites remain unknown, the results, obtained in terms of weight of 2,4 dichlorobenzophenone, were converted to the equivalent weight of o,p'DDD represented and are subsequently reported as milligrams of o,p'DDD in metabolized form.

Materials and methods

Extraction. Liquid specimens (serum, urine, stool emulsion, bile, and cerebrospinal fluid) were acidified to pH 2 and extracted with ether. Solid tissues, trimmed of fat and washed free of blood, were weighed and dried in a vacuum oven at 50° C. for 5 days. They were then reweighed and extracted with ether in a Bailey-Walker apparatus. The weights of the extracted residues thus allowed calculation of o,p'DDD concentrations both in terms of wet weight and of ether extractable material. The ether extracts of body fluids and tissues were

*Sheehan and colleagues[9] analyzed the fat of a patient who had been given DDD and also found a substance, other than DDD, with maximum absorption at 500 mμ.

†O, p' DDD (m.p. 77 to 79° C.) obtained from the Edcan Co., Norwalk, Conn., prepared by recrystallization from Rhothane (p, p'DDD with varying lesser amounts of o,p'DDD).

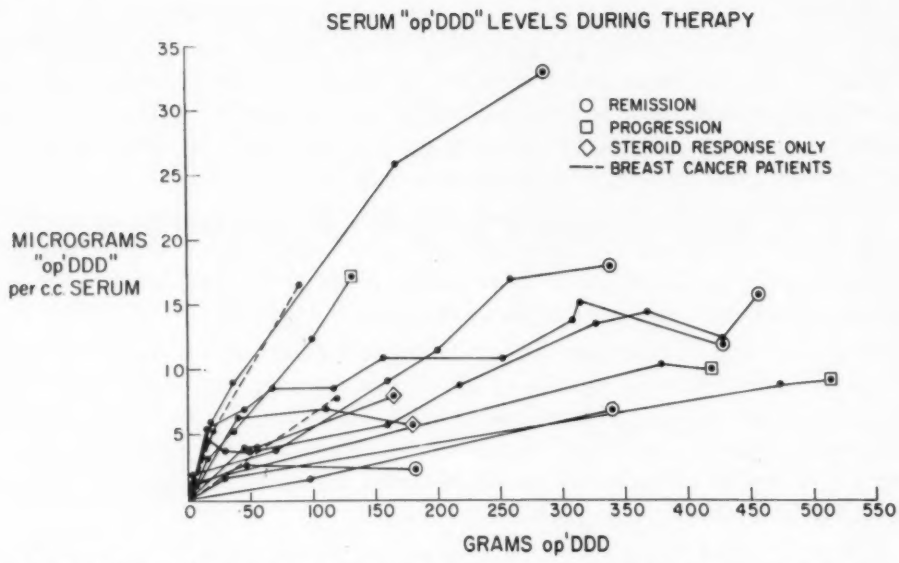

Fig. 1. Each line represents a single patient and the points are serial determinations of serum o,p'DDD. The figure on the last point signifies the patient's clinical result.

then washed with 0.5 N NaOH, according to the method of Ofner and Calvary,[5] to extract alkaline soluble metabolites. Appropriate aliquots of these ether and aqueous extracts of each tissue were then subjected to quantitative determination.

Clinical material. The patients ranged in age from 8 to 56 years and all except 2, who had metastatic cancer of the breast, had metastatic adrenal cancer. The usual mode of administration of o,p'DDD was by mouth in 0.5 Gm. capsules of the dry powder. Tablets of o,p'DDD and solutions in corn oil and oral Lipomul* were tried briefly, without apparent therapeutic advantage. A number of these patients also were given courses of intravenous o,p'DDD, up to 5 Gm. daily, in a Lipomul suspension. The usual oral dose was 10 Gm. daily.

It was frequently necessary to stop or lower the dosage of o,p'DDD because of side effects of nausea, vomiting, lethargy, and skin rash, and because of changes in the patients' clinical status. This unfortunate lack of standardization must be borne in mind in interpreting the results reported below.

Results

Absorption. Seven day stool collections of 3 patients who were able to maintain steady intake of o,p'DDD by mouth were analyzed. The patients were receiving 5, 10, and 15 Gm. per day, respectively, and were found to pass 66.5, 63.2, and 62.0 per cent, respectively, of the ingested drug, apparently unchanged, in the stool. This can explain in part the massive oral doses we have found it necessary to use. Since no o,p'DDD as such was identified in bile, it is unlikely that any of the drug found in the stool had contributed to the therapy of the patient.

Support for this absorption data is found in the urinary excretion studies. Patients who received the drug intravenously excreted the equivalent of about 25 per cent of their daily dose as urinary metabolite over 24 hours. Patients

*The Upjohn Co., Kalamazoo, Mich.

treated orally, however, were found to have a urinary metabolite excretion averaging about 10 per cent of their daily dose. When one assumes that, at most, about 40 per cent of this oral dose was absorbed, these excretion results become quite comparable.

Stool examinations made of one patient following the cessation of therapy revealed that most of the o,p'DDD cleared from the stool over the next 4 days. An average of 50 mg. per day was excreted in the pooled specimen of the second week, and an average of less than 1 mg. per day was recovered in the specimen collected during the third week after therapy was stopped.

Blood levels. Serum specimens were analyzed for o,p'DDD during various phases of therapy. Fig. 1 shows the changes in serum, o,p'DDD concentration of 13 patients following the onset of therapy. While the serum levels tended to rise during treatment in each patient, the rate of rise and the levels obtained were quite variable. Since most of the patients were able to maintain a dose of approximately 10 Gm. per day during this stage of treatment, the figure looks essentially the same when plotted against days insteads of against grams of o,p'DDD.

The blood levels were not necessarily higher in those patients showing toxic side effects and, as Fig. 1 shows, the absolute blood levels were not indicative of the patient's responsiveness to the drug. Serum levels tended to rise as long as intensive therapy was continued. The highest serum level obtained on oral dosage was 140 μg per milliliter after 1,900 Gm. o,p'DDD was given to a 10-year-old girl who did not respond to treatment.

When the drug was discontinued, serum levels fell, and in 4 patients who had received from 36 to 884 Gm. total dose they became undetectable, by the method used, after 6 to 9 weeks. The patient who received 1,900 Gm., however, showed a transient rise in serum level after the drug was stopped. She was anorectic and losing weight during this time, which may have resulted in more rapid mobilization of o,p'DDD from the fat stores. Two and one-half months later, the serum level of this patient was 60 μg per milliliter, or about 43 per cent of what it had been at the time treatment was discontinued.

Table I. Serum levels of o,p'DDD and its metabolite done on the same specimen at a time when the therapeutic result was apparent (metabolite weights are given as equivalent micrograms of o,p'DDD).

Serum	*o,p'DDD (μg/ml.)*	*Metabolite (μg/ml.)*	*M./o,p'DDD (%)*	*Therapeutic result*
1	7	29	415	Remission
2	11	38	345	No response
3	14	30	214	Steroid remission
4	17	37	218	Remission
5	19	30	158	No response
6	21	39	185	Remission
7	54	54	100	Remission
8	89	48	54	No response

The chromogen resulting from the metabolism of o,p'DDD was detected in all serum specimens examined. Table I shows the results of quantitative determination of both o,p'DDD and this metabolite in sera taken from 8 patients after the results of therapy were apparent and had been ranked in order of increasing o,p'DDD content. Like the o,p'DDD concentrations, there was no obvious relationship of the metabolite content to the patient's response. Again,

Table II. The concentrations of o,p'DDD in subcutaneous abdominal fat in terms of net weight and ether extractable lipid. Also shown is the intensity of drug administration

Patient	*Age (in years) and sex*	*Source*	*Total Gm. o,p'DDD*	*Days of therapy*	*Days since last dose*	*Milligram per Gm. wet weight*	*o,p'DDD per Gm. fat extractable*
1A	47, F	Biopsy	900	117	100	1.43	1.86
1B		Autopsy	700	100	5	2.68	3.72
2	35, F	Autopsy	320	33	0	1.27	1.42
3	49, F	Autopsy	210	24	5	1.73	1.95
4	32, F	Autopsy	410	55	5	3.49	5.78
5	51, M	Autopsy	120	23	15	0.46	0.76
6	51, M	Biopsy	250	30	60	0.49	0.60
7A	10, F	Biopsy	1,500 to	160	0	10.1	11.3
7B		Autopsy	1,900	210	110	8.75	10.3
8A	8, M	Biopsy	90 to	35	1	1.69	2.59
8B		Autopsy	310	80	1	2.84	3.76
9*	56, F	Biopsy	170	18	3	1.35	1.57
10*	54, F	Biopsy	130	14	6	1.38	1.83

*Breast cancer.

Table III. The concentrations of o,p'DDD in 5 tissues of 7 cases brought to autopsy. Table II shows the relationship of dosage to time of autopsy (micrograms of o,p'DDD per gram wet tissue weight)

Patient	*Total dose (Gm.)*	*Fat*	*Tumor*	*Adrenal*	*Brain*	*Liver*	*Last serum (μg/ml.)*	*Response*
1	700	2,680	145	763	39	187	19	Remission
2	320	1,270	49	0*	18	29	8	Steroid only
3	210	1,730	114	209	13	444	6	Steroid only
4	410	3,490	167	987	48	15	15	Steroid only
5	120	460	9	114	5	18	3	Remission
7	1,900	8,750	915	†	†	99	60	None
8	310	2,840	458	427	107	440	†	Steroid only

*Infarcted.
†Not available.

it must be borne in mind that several metabolites may be represented by this chromogen and also that the results reported in the table are in terms of the amount of o,p'DDD represented by the metabolite.

Tissue levels. Table II shows the o,p'DDD concentration in 13 specimens of subcutaneous abdominal fat from 10 patients. In addition are shown the total dose given to the patient, the duration of therapy, and the number of days that elapsed from the time that treatment was discontinued to the time that the specimen was obtained.

Of special interest are the 3 patients in whom biopsy as well as postmortem tissue was available. Patient No. 1 was given 2 courses of o,p'DDD separated from one another by 14 months; regression of disease occurred both times, with progression in the interim. While the higher fat concentration of o,p'DDD following the second, and somewhat smaller, course of drug could represent an addition to the levels in the tissues remaining from the course given 14 months earlier, it is more likely that the higher concentration reflects the shorter delay between the end of the second course and obtaining the specimen. That this patient was clearing o,p'DDD from the tissues following the first course is evidenced by the fact that 30 days after this course was discontinued the equivalent of 414 mg. o,p'DDD was excreted in a 24 hour urine collection. A similar picture is seen in Patient No. 7, who had the highest tissue concentrations seen. The biopsy specimen taken during treatment after 1,500 Gm. had been given contained a higher concentration of o,p'DDD than the postmortem specimen obtained after the end of the 1,900 Gm. course. There was, however, a period of 110 days between the end of the course and the death of the patient. Tissues were obtained from Patient No. 8 during and immediately following his course and, in this case, rising tissue levels are seen.

Of the 8 patients listed with adrenal cancer, Patients Nos. 1 and 5 had shown regression of tumor and died of other causes. Patient No. 7 died of advancing disease without response. The remaining 5 patients all had shown at least a 70 per cent drop in tumor steroids but died of various causes during treatment; Patient No. 2 died during an episode associated with massive tumor necrosis,[3] Patient No. 3 died of complications of the tumor, Patient No. 4 died postoperatively, having had a complete steroid remission with a previous course, Patient No. 6 died at home of unknown causes, and Patient No. 8 died of an unrelated tumor, namely, an astrocytoma.

Table III shows the concentrations of o,p'DDD in fat, adrenal, tumor, liver, and brain of the 7 cases of adrenal cancer that were brought to autopsy. Though the data are limited, the information in Tables II and III make it doubtful that tissue levels of o,p'DDD will be sufficient to explain the response of these patients; noteworthy is the contrast between Patient No. 5, who responded, and Patient No. 7, who did not.

Following their courses of o,p'DDD, Patients Nos. 9 and 10, who had breast cancer, continued to have blood and urinary 17 OH corticoids in the normal range but showed complete lack of responsiveness to 40 μ of ACTH given intravenously over 8 hours. The adrenal o,p'DDD concentrations for these 2 patients were 0.24 mg. and 0.14 mg. per gram wet weight, respectively.

Table IV shows the concentrations of o,p'DDD and its metabolite in 8 tissues of one patient (No. 1). With the exception of brain, the o,p'DDD concentrations are somewhat less variable when expressed in terms of tissue fat (ether extractable material) than in terms of wet weight. The opposite appears to be true for the metabolite. This may well reflect their lipid and water solubility, respectively. Other tissues examined for o,p'DDD concentrations are shown in Table V.

Two specimens of cerebrospinal fluid revealed no detectable o,p'DDD and only 3 and 4 μg per milliliter as metabolite. Bile likewise contained no detectable o,p'DDD but did contain sufficient concentrations of metabolite chromogen to make this a significant mode of excretion, as is pointed out below.

Table IV. The concentrations in 8 tissues from one patient (No. 1, F. F., adrenal cancer) of o,p'DDD and its metabolite both in terms of wet weight and of ether extractable lipid

	μg o,p'DDD		*Metabolite as μg o,p'DDD*	
Tissue	*Per Gm. wet weight*	*Per Gm. fat*	*Per Gm. wet weight*	*Per Gm. fat*
Fat	2,680	3,720	22	39
Tumor	145	1,850	27	350
Adrenal	763	3,020	60	240
Brain	39	275	11	81
Liver	187	1,550	87	720
Kidney	38	2,590	18	1,260
Muscle	142	3,310	10	260
Heart	121	6,920	3	194

Table V. Content of o,p'DDD in other tissues of Patient No. 1 (total dose, 700 Gm.)*

Tissue	*μg o,p'DDD/Gm. wet weight*
Marrow (spine)	318
Nerve (sciatic)	122
Skin (abscessed)	75
Uterus	73
Pancreas	66
Pituitary	59
Bowel (small intestine)	58
Bladder	44
Gall bladder	24
Thyroid	28
Lung	28
Breast	22
Spleen	16
Ovary	14
Testis (from Patient No. 5, total dose, 120 Gm.)	12

*Other tissues of this patient are shown in Table IV.

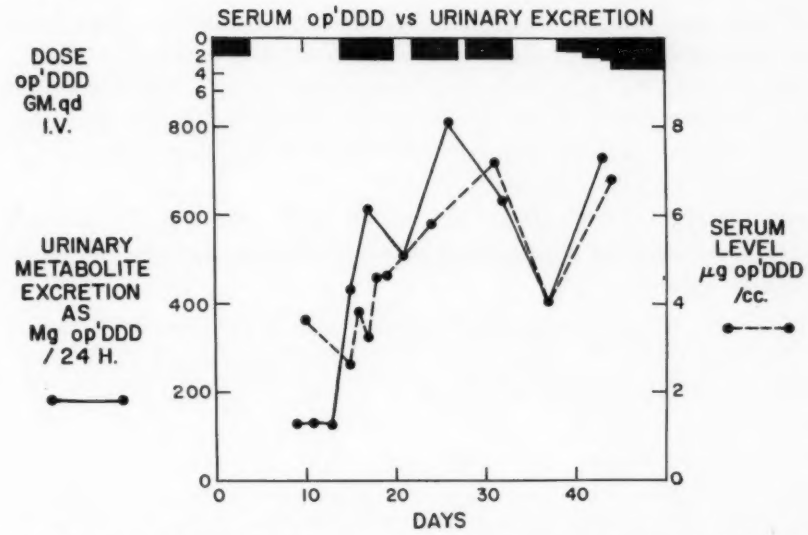

Fig. 2. The relationship of serum o,p'DDD and urinary metabolic chromogen to the intravenous administration of o,p'DDD. The urinary chromogen (metabolite or metabolites of o,p'DDD) are graphed as equivalent weight of o,p'DDD. O,p'DDD as such did not appear in the urine.

Excretion. Similar to its related compounds, o,p'DDD appears to be metabolized to a water soluble form (possibly o,p'dichlorodiphenyl acetic acid) and excreted in the urine. The greatest amount determined in a 24 hour urine specimen was the equivalent of 1.3 Gm. o,p'DDD in a patient receiving 10 Gm. daily by mouth. The amount excreted daily rose during early treatment and, from 10 urine samples of patients able to maintain a steady dose, plateaued at an average of about 10 per cent (7.8 to 13.0) of the daily oral dose. Ten urine specimens of patients on steady intravenous o,p'DDD averaged about 25 per cent (18.2 to 32.0) of the administered dose. As mentioned above, this discrepancy can be accounted for when it is considered that only about 35 to 40 per cent of the oral dose is absorbed.

Similar to the blood and tissue levels of o,p'DDD, the urine concentrations of metabolite chromogen fell rather slowly after the course had been stopped. Values during the first week after an intensive course showed little change. One month following their courses, Patients Nos. 1 and 3 were found to be excreting 414 and 594 mg. per 24 hours, respectively.

Fig. 2 shows the relationship between blood levels of o,p'DDD and the urinary levels of metabolite chromogen in a patient receiving the drug intravenously. It can be seen that they tend to run a parallel, somewhat delayed, response to the administered dosage.

A second mode of excretion, though, from the limited data available, more variable from patient to patient, is via the biliary tract. Three samples of gall bladder bile were analyzed and, as stated above, contained no detectable o,p'DDD but did contain the equivalent of 0.33, 0.47, and 2.17 mg. per milliliter of o,p'DDD as metabolite chromogen.

Four 7 day stool collections of patients on steady doses of drug were extracted for the metabolite and were found to contain the equivalent of 1.0, 1.5, 4.5, and 17.0 per cent of the dose given during that period. The patient whose biliary concentration was 0.47 mg. per milliliter excreted 1.5 per cent and the patient whose biliary concentration was 2.17 mg. per milliliter excreted 4.5 per cent of their daily doses by this route.

Thus, considering the ranges of absorption and excretion, the patients in this study were apparently retaining from 20 to 30 per cent of the oral dose administered each day.

The author wishes to acknowledge his indebtedness to the late Dr. Delbert M. Bergenstal, and to Drs. Mortimer B. Lipsett, Roy Hertz, and John Weisburger for their advice and encouragement in this study, and to Messrs. Wilbert Nixon and Fred Dhyse for their technical assistance.

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Passage of penicillin across the blood-brain barrier in adults

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The use of intraspinal penicillin in the management of pneumococcic meningitis remains a controversial issue. Some authorities continue to recommend its use initially in the treatment of this disease, the rationale being to establish immediate bactericidal levels in the cerebrospinal fluid. Although the small doses presently recommended have resulted in very few complications, when mistakes are made in the dose administered, results may be fatal.

The purpose of this study was to determine if prompt bactericidal levels of penicillin could be obtained in the cerebrospinal fluid if the initial amount of parenteral penicillin given was increased above the dose range presently recommended.

A total of 24 patients without inflammatory disease of the central nervous system was studied. Crystalline penicillin was administered in the dosage range of 4 to 5 million units by both the intramuscular and intravenous routes. Samples of cerebrospinal fluids were obtained from the lumbar subarachnoid space at different time intervals and the concentration of penicillin was determined.

The results of this study indicate that when 5 million units of crystalline penicillin are administered intravenously in a 3 to 5 minute period, a rapid, effective, and safe method is available for obtaining bactericidal concentrations of this drug in the cerebrospinal fluid; thus the use of intraspinal penicillin can be eliminated justifiably.

Since the introduction of antibiotics into clinical medicine there has been much discussion and debate regarding the most efficient antibiotic program to institute in the management of patients with purulent meningitis. Initially, intraspinal penicillin was considered the treatment of choice; however, there arose a fairly large number of complications as a direct result

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The contents of this article are the personal views of the author and are not to be construed as statement of official Air Force policy.
Received for publication Jan. 16, 1961.

of this route of administration.[1-3] Dowling and associates[4] were the first to report the relatively successful treatment of pneumococcic meningitis with "large" doses of parenterally administered penicillin, and it was only after that publication that most authorities discontinued the use of repeated injections of intraspinal penicillin.

In the above-mentioned study[4] it was found that there was a delay period of approximately 6 to 8 hours before bactericidal concentration of penicillin could be recovered from the cerebrospinal fluid; therefore, it was felt that an initial, single small intraspinal dose of this drug was probably indicated in the treatment of pneumococcic meningitis in order to establish immediate bactericidal levels during this lag phase.

At the present time, some 10 years later, the need for intraspinal penicillin as an initial therapeutic measure in pneumococcic meningitis remains a controversial issue.[5-9] Intraspinal penicillin in the small doses presently recommended (5 to 10 thousand units) in itself has resulted in very few complications[9]; however, when mistakes are made in the dose administered, as we have recently encountered at this hospital,[10] the results may be fatal. We have also verbal reports from 2 other university hospitals of deaths resulting from similar mistakes. In addition to this hazard, the introduction of a foreign material into the subarachnoid space also carries a certain risk.

The purpose of this study was to determine if prompt bactericidal concentrations of penicillin could be obtained in the cerebrospinal fluid if the initial amount of parenterally administered penicillin was increased above the dose range presently recommended.

Methods

A total of 24 patients without inflammation of the meninges were studied with regard to the rate of recovery and concentration of penicillin that could be obtained from the lumbar subarachnoid space following the administration of a crystalline penicillin by the intramuscular and intravenous routes. The patients selected for this study were volunteers on whom a lumbar puncture was to be performed for a diagnostic purpose or to whom penicillin was to be given for a specific purpose. These patients had not received any antibiotics for a period of at least 4 months prior to this study. The age range of the patients studied was from 32 to 80 years; there were 14 men and 10 women. The primary diagnoses of the patients studied included cerebral arterial thrombosis, rheumatic heart disease, carcinomas of the breast, lung, and rectum, psychoneurosis, fracture of the femur, vitamin deficiency, peptic ulcer, portal cirrhosis, and hypertension (see Tables I, II, and III for details). All patients had normal cerebrospinal fluid pressures and normal protein and cell counts.

Four or 5 million units of crystalline penicillin were given intramuscularly into the gluteal region and samples of cerebrospinal fluid were obtained for the determination of penicillin concentration at intervals of one-half and 1 hour. An identical amount of aqueous penicillin was dissolved in 150 ml. of 5 per cent dextrose and water and given intravenously over a 3 to 5 minute period. Samples of cerebrospinal fluid were collected at 15 minute, one-half hour, and 1 hour intervals. All samples of cerebrospinal fluid were obtained from the lumbar subarachnoid space. A review of the literature failed to reveal any data on the possible complication that might be encountered when intravenous penicillin in this dose range is administered over this short period; however, one paper suggests that in the presence of renal disease the intravenous administration of 12 million units of penicillin as often as every 4 hours may be fatal.[11] The patients in the present study were observed very closely and no immediate or late complications were encountered.

Table I. Group I

Age (in years), race, and sex	*Diagnosis*	*Amount (units)*	*Penicillin*	*Concentration in cerebrospinal fluid (unit/ml.)*	
				½ hour	*1 hour*
69 C, M	Cerebrovascular thrombosis	4 million	I. M.	.04	.04
80 N, M	Cerebrovascular thrombosis	4 million	I. M.	.02	.02
46 C, F	Rheumatic heart disease	4 million	I. M.	.04	.04
62 N, F	Breast carcinoma	5 million	I. M.	.02	.02

Crystalline penicillin G was used in both the in vitro control measurements and the patient study. Penicillin concentrations were determined by the dilution method of Rammelkamp,[12, 13] using a strain of alpha hemolytic streptococci as the test organism. Complete sterilization of the test culture took place at concentrations of 0.1560 or 0.0780 unit of penicillin per milliliter, which is only a 1 tube dilution difference. Each study was set up with a control and final calculations were made according to the control for that day. Since Appelbaum and colleagues[14] have shown that, of 75 strains of pneumococci tested, all were sensitive to concentrations of penicillin above 0.06 unit per milliliter, and only 3 strains were able to survive at concentrations between 0.02 and 0.06 unit per milliliter, the lowest concentration of penicillin tested for was 0.02 unit per milliliter.

Results

The patients studied were divided into 3 groups, depending on the route and dose of penicillin administered. Group I (Table I) consisted of 4 patients who received crystalline penicillin by the intramuscular route in doses of 4 and 5 million units. Samples of spinal fluid were withdrawn 30 minutes and 1 hour after the drug was administered as described. In this group the concentrations of penicillin recovered from cerebrospinal fluid were less than 0.02 and 0.04 unit per milliliter.

Group II (Table II) consisted of 10 patients who received 4 million units of penicillin intravenously over a 3 to 5 minute period. Thirty minutes after the penicillin had been administered, 8 of the 10 patients had penicillin levels above the minimum bactericidal concentration for pneumococci (0.06 unit per milliliter), and within 1 hour all of this group were above this level.

Table II. Group II

Age (in years), race, and sex	*Diagnosis*	*Amount (units)*	*Penicillin*	*Concentration in cerebrospinal fluid (units/ml.)*				
				½ hour	*1 hour*	*2 hours*	*4 hours*	*6 hours*
40 C, M	Psychoneurosis	4 million	I. V.	.08	.31			
73 C, F	Hip fracture	4 million	I. V.	2.5	2.5			
55 N, F	Breast carcinoma	4 million	I. V.	.31	.31			
67 N, F	Peptic ulcer	4 million	I. V.	.16	.16			
32 C, F	Psychoneurosis	4 million	I. V.	.16	.16			
80 N, M	Cerebrovascular thrombosis	4 million	I. V.	.08	.16			
52 C, F	Hypertension	4 million	I. V.	.04	.08			
38 N, M	Vitamin deficiency	4 million	I. V.	.08	.08			
45 C, M	Psychoneurosis	4 million	I. V.	.02	.08			
66 W, M	Cerebrovascular thrombosis	4 million	I. V.	.08	.16	.16	.08	.08

Table III. Group III

Age (in years), race, and sex	*Diagnosis*	*Amount (units)*	*Penicillin*	*Concentration in cerebrospinal fluid*		
				15 minutes	*½ hour*	*1 hour*
67 C, M	Cirrhosis	5 million	I.V.		.32	.64
80 N, M	Lung carcinoma	5 million	I.V.		1.3	1.3
62 N, M	Lung carcinoma	5 million	I.V.		.32	.64
65 N, F	Cerebrosvascular thrombosis	5 million	I.V.		.08	.08
46 N, M	Rectum carcinoma	5 million	I.V.		.08	.08
70 C, F	Cerebrovascular thrombosis	5 million	I.V.		.08	.16
72 N, F	Breast carcinoma	5 million	I.V.	.08	.16	
80 N, M	Cerebrovascular thrombosis	5 million	I.V.	.08	.16	
45 F	Cervix carcinoma	5 million	I.V.	.04	.16	
67 M	Cerebrovascular thrombosis	5 million	I.V.	.04	.16	

In Group III (Table III) the amount of penicillin given by the intravenous route was increased to 5 million units, and it was administered over the same period of time. All of the patients had bactericidal concentrations of this drug in the lumbar cerebrospinal fluid within 30 minutes. Four of this group were studied 15 minutes after the penicillin was administered; 2 had 0.04 and 2 had 0.08 unit per milliliter. When these results were subjected to analysis, there was no significant correlation between the concentration of penicillin recovered and the age, weight, or underlying disease of the patients studied.

Discussion

The use of intraspinal penicillin as an initial therapeutic measure in pneumococcic meningitis remains a somewhat controversial issue. Some believe this mode of therapy is indicated on the basis of better clinical results[7, 9]; others contend equally as strong that it is not only unnecessary, but dangerous.[1, 3, 5, 8, 15, 16] Since there have been no controlled studies with this question in mind, it is impossible to settle this issue on purely clinical grounds. In a recent report on the treatment of pneumococcic meningitis,[17] in which intraspinal therapy was not used, the mortality rate was lower than that obtained by a group which recommends the continued use of intraspinal penicillin.[9]

Intraspinal penicillin has caused very few complications when administered in the small doses presently recommended; however, when mistakes are made in the amount given, the results may be fatal.[10] Even when penicillin is administered by this route, it has been reported that only trace quantities occasionally could be recovered from the lateral ventricles in a period of 2 hours following the administration of 75 hundred units of this drug into the lumbar subarachnoid space.[18] This delayed and unequal distribution of penicillin in the cerebrospinal fluid is not therapeutically ideal in this illness.

As yet, we have not had the opportunity to study patients with purulent meningitis; however, it has been shown by others that penicillin crosses the blood-brain barrier much more rapidly, and at greater concentrations, when there is inflammation of the meninges.[19, 20] Also pertinent to the study is the fact that, following the administration of parenteral penicillin, the concentration of this drug in the cerebrospinal fluid increases as one progresses cephalad in the subarachnoid space[21]; thus, the concentrations of penicillin which were recovered from the lumbar region in our study represent the lowest concentration of pencillin in this fluid-containing cavity.

Verron[22] studied the passage of several penicillin preparations into the cerebrospinal fluid following the intramuscular administration in children. After penicillin G sodium was given in the dose of 50,000 units per kilogram of body weight, it was reported that, on the average, approximately 0.04 unit per milliliter of this drug could be recovered from the lumbar subarachnoid space 30 minutes and 0.16 unit per milliliter one hour following its administration. From our study in adults, with penicillin G potassium administered in the same fashion and at comparable doses, the results of the above study could not be duplicated. It was only after the penicillin was administered by the intravenous route that prompt bactericidal concentration could be obtained.

In view of these studies, it would appear that the only time intraspinal penicillin therapy would have a place in the management of purulent meningitis would be in a patient who presented with hypotension as part of the clinical picture, since an inadequate blood flow may delay the passage of this drug across the blood-brain barrier.

The author gratefully acknowledges the advice and criticism given by Drs. William Parson, Thomas Hunter, William Jordan, T. R. Johns, and K. Russel, and the technical assistance given by Carrol Gordon.

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A simple method of blood volume determination for clinical use

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A method is described for blood volume determination utilizing the hemoglobin concentration changes following the infusion of Intradex in normal saline. The test requires no special equipment or training beyond that normally available in all hospitals. Experience with this blood volume method leads us to suggest that it yields information of sufficient accuracy for clinical purposes. The technique of the application of this method to blood replacement therapy is presented.

The clinical management of obvious or questionable hemorrhage, where rapid blood replacement seems indicated, can be difficult. In this situation, because the amount of blood loss is difficult to predict from clinical signs, information regarding the immediate blood volume status can be of great value. The standard methods available for estimating blood volume,[1, 2] such as the radioactive labeled compounds and dye dilution methods, require specialized equipment and well-trained technical personnel. This tends to limit the availability of these methods to the clinician. The objective of the present study was the development of a blood volume method which could be performed without special training or equipment and yet be sufficiently accurate for clinical use. Such a method should be useful in hospitals where other methods are not available, or under field conditions. This report contains a description of a method which seems to meet these requirements and outlines briefly its application to blood replacement therapy.

Materials and methods

Blood volume determination.

A. PRINCIPLE. The method is based on the dilution principle, utilizing the changes in hemoglobin concentration following an infusion of a plasma volume substitute. By means of

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Received for publication Aug. 1, 1960.

a photometric method it is possible to obtain the concentration in grams per cent of a colored solute in an aqueous solution. When water is added to the solution, it is obvious that the concentration of the solute in solution will be decreased in direct proportion to the volume of water added. If the initial volume of the solution and the concentration of the solute in the solution are known, the final concentration, after a known volume of water has been added, can be calculated. Conversely, if the initial and the final concentrations of the solute in the solution and the volume of the added water are known, then it is possible to calculate the initial volume of this solution.

In the method to be described the initial and final concentrations of the solute are the initial and final hemoglobin concentrations, and the volume of the added solution is a known amount of 6 per cent Intradex* in normal saline infused intravenously. Applying the previously stated principle, it is then possible to calculate the initial volume of the solution (the preinfusion blood volume), providing that there is a temporary expansion of the circulating blood volume by approximately the same volume as the infused volume of the fluid and that there is a complete mixing of the infused fluid with the circulating blood.

B. METHOD. 1. Clinical shock should be alleviated by appropriate means before the test is started and an interval of 45 to 60 minutes should be allowed between the start of the test and the time at which all other parenteral fluids are discontinued or at least slowed to a rate of 10 drops per minute or less.

2. A blood sample (approximately 5 ml.) from a large vein in the anticubital fossa of the arm is oxalated with dry ammonium–potassium oxalate powder (Hgb_i). The tourniquet applied to the arm for venipunctures should be in place for only a short time to avoid hemoconcentration distal to it.

3. Immediately following the above step, using a No. 18 needle, 500 ml. of 6 per cent Intradex in normal saline (hereafter referred to as Intradex) is infused intravenously at a uniform rate in 20 to 30 minutes (unless there are special contraindications).

4. After the infusion is completed, 5 ml. specimens are taken for hemoglobin determinations from a large anticubital vein of the opposite arm (to avoid artefactual dilution). The first specimen is taken immediately at the completion of the infusion and subsequent specimens at precisely 10, 20, and 30 minutes (Hgb_f).

5. After all blood specimens have been obtained, the hemoglobin concentration of each of the 5 specimens is determined in triplicate by a photometric method, in immediate succession by the same person. The following points in technique must be strictly adhered to: (A) The same hemoglobin pipette must be used for all determinations (individual hemoglobin pipettes may vary ± 5 per cent[2, B]; (B) Blood specimens must be thoroughly mixed just prior to pipetting because dextran increases the sedimentation rate; (C) Hemoglobin diluent must be freshly pipetted (using the same pipette throughout) for each determination, to avoid evaporation; (D) The photometer must be accurately calibrated (most are accurate between 4.2 and 19 Gm. per cent of hemoglobin[4]).

6. The blood volume prior to the infusion can then be calculated by the use of the following formula derived from the principle:

$$(V_i)\ (Hgb_i) = (V_i + V_k)\ (Hgb_f);\ \text{or}\ V_i = \frac{(V_k)\ (Hgb_f)}{(Hgb_i - Hgb_f)}$$

where V_i = the unknown initial blood volume at the moment prior to the infusion, Hgb_i = the initial hemoglobin concentration determined on the specimen obtained before the infusion, V_k = the known volume of the infused 6 per cent Intradex, and Hgb_f = the final hemoglobin concentration. This value is the lowest hemoglobin concentration found in the specimens taken during the 30 minutes after the infusion has been completed. Technical error should be suspected if the curve of these serial postinfusion blood specimens is not smooth.

For example, a normal subject was given 500 ml. of 6 per cent Intradex in normal saline solution intravenously in 20 minutes and the data in Table I were obtained.

*The Intradex (6 per cent dextran solution in normal saline) used in this study was supplied by Glaxo (Can.) Ltd., Toronto, Ont.

Table I

Measured material	*Minutes before infusion*	*Minutes after infusion*			
		0	*10*	*20*	*30*
Hemoglobin (Gm. %)	12.8	11.8	11.4	11.4	11.4

Calculation.

$$Hgb_i = 12.8 \text{ Gm. \%} : V_k = 500 \text{ ml.} : Hgb_f = 11.4 \text{ Gm. \%}$$

$$\text{Since } V_i = \frac{V_k(Hgb_f)}{(Hgb_i - Hgb_f)} \text{ or } \frac{(500)\ (11.4)}{(12.8 - 11.4)}$$

Therefore, V_i = approximately 4,100 ml.

Results

Table II shows the results of the described method in a group of normal control subjects with no history of recent bleeding. These results are compared with the volume estimated on a weight basis and in 4 of these subjects with the volume as determined by the radioactive iodinated serum albumin method.

In a series of 35 patients with definite hemorrhage, transfusion of the estimated blood loss returned the hemoglobin concentration to a normal level. In a number of patients with questionable internal hemorrhage, the results of the test were confirmed by subsequent events. A few cases are presented in the *Appendix* to illustrate results obtained in clinical application of the method.

Application of method to blood replacement therapy.

A. BLOOD VOLUME DEFICIT. The blood volume deficit after hemorrhage is the difference between the blood volume prehemorrhage and that measured post-hemorrhage. As the prehemorrhage blood volume most likely will not have been measured, it has to be estimated by some indirect means, based on weight, height, or surface area. The volume for the average normal adult man has

Table II. Blood volume determinations in normal subjects

Case No.	*Sex*	*Age (years)*	*Weight (pounds)*	*Hgb_i*	*Hgb_f*	*B.V.D.*	*B.V.W.*	*Blood volume by R.I.S.A. method*
1	M	22	120	15.2	13.6	4,200	4,000-4,400	4,000 ml.
2	M	20	116	16.0	14.2	4,000	3,800-4,300	4,750 ml.
3	M		130	15.4	14.2	4,500	4,300-4,800	4,800 ml.
4	M	18	156	15.4	14.2	5,900	5,100-5,800	5,200 ml.
5	F	68	103	13.0	11.4	3,600	3,100-3,600	
6	M	58	118	14.6	13.0	4,100	3,900-4,400	
7	F	26	118	12.8	11.4	4,100	3,500-4,100	

Abbreviations: Hgb_i = Pre-dextran infusion hemoglobin concentration gram per cent.
Hgb_f = Lowest hemoglobin concentration gram per cent in the 30 minute period following the infusion of 500 ml. of 6 per cent Intradex in 20 minutes.
B.V.D. = Blood volume in milliliters, as determined by the described method.
B.V.W. = Blood volume in milliliters, estimated from weight using the range of 33 to 37 ml. per pound for men and 30 to 35 ml. per pound for women.[1,B]
R.I.S.A. = Radioactive iodinated serum albumin.

been shown to be approximately 76.6 ml. per kilogram, or 35 ml. per pound.[1, B] It is less in the woman than in the man, less in the elderly than the young, and less in the obese than the lean.[5] In this study the range used for men was 33 ml. to 37 ml. per pound and for women 30 ml. to 35 ml. per pound, with the lower figure for the elderly or obese, or for those with a history of heart disease, congestive heart failure, or chronic anemia. The difference between the estimated blood volume by weight and the posthemorrhage blood volume by determination will approximate the blood volume deficit.

B. BLOOD LOSS CORRECTED BY HEMODILUTION. The blood volume deficit will not necessarily reflect the total amount of whole blood that was lost. When blood is rapidly lost by hemorrhage, there is a proportional decrease in the blood volume, and as it is whole blood that is lost, the hemoglobin concentration and the hematocrit will be at the most only slightly altered during this initial period. However, provided that the patient is not markedly dehydrated, when the blood volume becomes decreased, a gradual absorption from the extravascular fluid occurs, so that the plasma volume, and thus the blood volume, increases toward normal. This relative increase in the plasma volume over the red cell volume, known as hemodilution, leads to a corresponding decrease in the hemoglobin concentration. According to Mollison[1, C] a loss of approximately 1,000 ml. of whole blood in a normal man requires 36 to 72 hours for complete

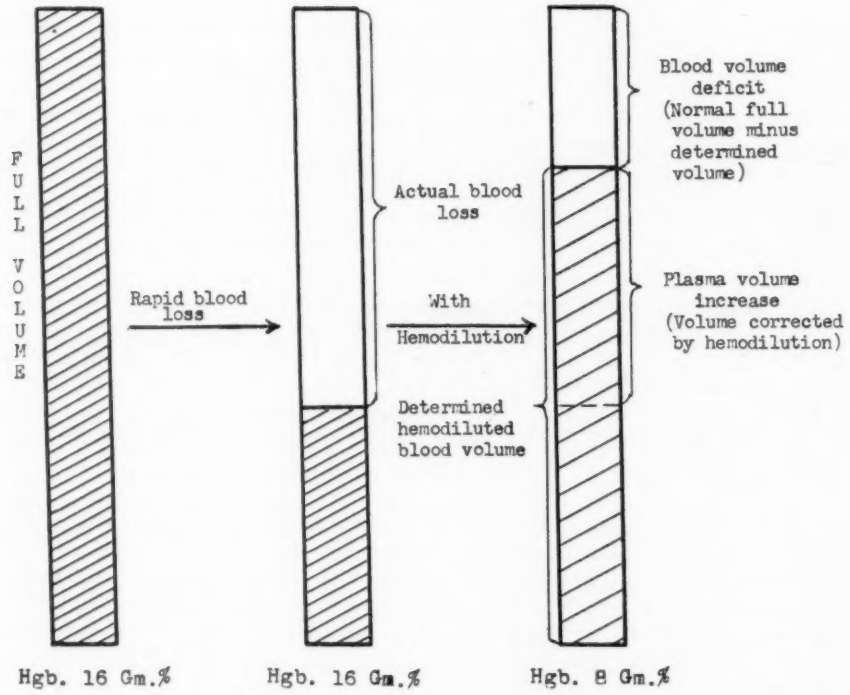

Fig. 1. Changes in the blood and plasma volume and hemoglobin concentration following rapid blood loss.

hemodilution to full volume. The total amount of whole blood lost consists of the sum of the measured blood volume deficit plus the amount of plasma volume increase (resulting from the hemodilution process) represented in the measured blood volume (Fig. 1).

The plasma volume increase caused by hemodilution is the difference between the measured (hemodiluted) blood volume and the volume of nonhemodiluted blood with the normal hemoglobin concentration present in this measured blood volume. The volume of nonhemodiluted blood can be calculated by division of the total amount of hemoglobin in the hemodiluted blood by the normal hemoglobin concentration. So, the volume of nonhemodiluted blood $=$

$$\frac{\text{determined hemodiluted blood volume} \times \text{hemodiluted hemoglobin concentration}}{\text{normal hemoglobin concentration}}$$

For example, if, after hemorrhage, there is a hemoglobin concentration of 10 Gm. per cent, a determined blood volume of 3,000 ml., and an estimated normal blood volume by weight of about 5,000 ml., then the measured blood volume deficit is 5,000 – 3,000, or 2,000 ml. Assuming the prehemorrhage hemoglobin concentration to have been 15 Gm. per cent, then the amount of blood loss corrected by hemodilution was as follows: B.V.D. – $\left(\text{B.V.D.} \times \frac{\text{hemodiluted hemoglobin concentration}}{\text{normal hemoglobin concentration}}\right)$ or 3,000 ml. – $\left(3{,}000 \text{ ml.} \times \frac{10 \text{ Gm. \%}}{15 \text{ Gm. \%}}\right)$, or 1,000 ml., and the total amount of whole blood lost was 2,000 ml. + 1,000 ml., or 3,000 ml. of whole blood of 15 Gm. per cent hemoglobin concentration. As the prehemorrhage hemoglobin concentration usually is not known, this is really the amount of blood of 15 Gm. per cent hemoglobin concentration required for return to full volume with a hemoglobin concentration of 15 Gm. per cent.

It is beyond the scope of this paper to enter into the details of replacement transfusion therapy. It should be noted, however, that the rapid replacement of the calculated total amount of blood loss (blood volume deficit plus amount corrected by hemodilution) to a patient with a hemodiluted blood volume would result in hypervolemia and could be very dangerous. We found it safe to replace the blood volume deficit by rapid transfusion of whole blood to 90 per cent of the estimated normal blood volume. The remainder of the total blood loss can then be corrected more slowly with either whole blood or packed red cells, with 24 or 48 hours between infusions to allow the volume compensating mechanisms to operate. Only the amount of whole blood which is transfused should be considered in blood replacement calculations. Preserved blood contains both whole blood and an anticoagulant solution, but, as the latter does not remain long in the circulation, it should not be included in the replacement calculations.

Discussion

The blood volume determined by the described method in a group of normal control subjects compared favorably with that estimated on a weight basis and with that determined simultaneously in 4 subjects by a radioactive iodinated serum albumin method (Table II). The results obtained with patients who

had bled were not compared with any other method. However, transfusion of the estimated blood loss returned the hemoglobin to a normal concentration, suggesting that the method is accurate enough for use in the clinical management of hemorrhage.

The principle of the described method depends on two assumptions. The first is that there is complete mixing of the infused material with the circulating blood. This assumption must be made for all blood volume methods in common use. As in all dilution methods, it is necessary to alleviate shock before performing the test, as it is otherwise impossible to be certain that complete mixing has occurred. In the present study the postinfusion hemoglobin concentration curve showed a gradual decline, reaching a plateau in 10 to 20 minutes, suggesting maximal mixing; this is in agreement with the time found for complete mixing to occur in other dilution methods. The second assumption is that the blood volume is temporarily expanded by a volume approximately equal to the volume of Intradex infused. This is more difficult to substantiate definitely. Vickery[6] and Bollman[7] found no histologic evidence of extravascular diffusion of dextran, except in pulmonary edema and severe burns. The results of studies relevant to volume increase after dextran infusion are difficult to assess.[8-15] Moore[16] seems to believe that the evidence is sufficient to suggest that dextran infusion results in a temporary increase in plasma volume equal to the volume infused. Koster and associates[17] found the plasma volume increase approximately equal to the amount of 6 per cent Macrodex in saline (average molecular weight 91,000) infused, but we were unable to find data regarding the volume increase after infusion of Intradex in saline (mean molecular weight 200,000)*[1, D] the solution used in the present study. In the presently described method the hemoglobin concentrations did not decrease to any extent after 10 minutes postinfusion, suggesting that during the period of the test no substantial increase occurred in the plasma volume beyond that of the volume of Intradex infused. Only Intradex in saline has been used with the described method and we have not assessed its applicability to dextrans of lower average molecular weight.

The patients tolerated the rapid infusion of Intradex in saline. Cases near clinical shock were usually improved by the infusion, which acts as an emergency treatment for hypovolemia while whole blood is being procured. In other words, at the same time that the test is being performed, treatment of the patient is being instituted. Studies have shown that dextran does not interfere with immunity, antibody formation, wound healing, or resistance to infection, and that reactions are relatively infrequent and mild.[18-20] A hemorrhagic tendency has been reported after the infusion of large amounts of dextran[21, 22] but we have not encountered this after the infusion of 500 ml. of Intradex. The infusion should be given with caution to those patients who are significantly anemic and to those with a history of myocardial infarct or congestive heart failure. With any infusion given at a time that active hemorrhage is occurring there is a strong possibility that part of the infused material will be lost from

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the vascular system. For this reason it is impossible to be certain of the results obtained if the test is performed while the patient is actively bleeding. This holds true for all blood volume methods utilizing the dilution principle.

Absolute hemoglobin concentration values are not the determining factor of the described method. It is a comparative method, the relative hemoglobin concentration changes postinfusion being compared with the preinfusion concentration, which is equivalent to a standard. At the beginning of this study both hemoglobin concentration and hematocrit determinations were carried out. However, it was found that the hematocrit determination was affected by the type and amount of anticoagulant used,[3, 4] and that dextran infusion tended to cause increased rouleau formation and increased mean corpuscular hemoglobin concentration. Merriman and colleagues[23] also noted the latter and suggested that dextran causes a shrinkage of the red blood corpuscles resulting in a fallaciously low hematocrit level. As the hemoglobin concentration was not affected by these changes, we have used it as the measure and have attempted to reduce its inherent error to a minimum by the precautions outlined. The accuracy of the method depends upon strict adherence to the details of the laboratory technique. The entire procedure can be performed in about 90 minutes. It requires only equipment and materials already present in all hospitals. Laboratory technicians and house officers easily learn the few extra details and quickly become proficient in performing the hemoglobin determinations.

Blood volume dilution methods utilizing changes in the hemoglobin concentration following plasma or packed red cell infusion have been previously described.[24-27] Sharpey-Schafer[24] found a method utilizing packed red cell infusion to agree within 500 ml. of the results of the Evans blue method. These reports did not specify whether serial postinfusion hemoglobin determinations were used, nor what precautions were taken to reduce the errors in the hemoglobin concentration determinations. Plasma and packed red cells have the advantage of being physiologic, but they are not as readily available as dextran, there is a danger of serum hepatitis or mistransfusion, and the volume of anticoagulant solution is not accurately known. With packed red cells there is also the possibility of immediate posttransfusion hemolysis of some of the red blood cells.

It is apparent from the principle of dilution that in the described method the smaller the preinfusion blood volume and/or the higher the initial hemoglobin concentration, the less small technical errors will affect the accuracy of the results. Conversely, in individuals with a circulating blood volume above 5,000 ml. and/or a very low preinfusion hemoglobin concentration, since only 500 ml. of Intradex is being infused, the fall in hemoglobin concentration will be quite small. With a fall of less than 1 Gm. per cent, small technical errors will result in a fallaciously high calculated blood volume. However, even in individuals whose normal blood volume is above 5,000 ml., any appreciable hemorrhage will have reduced the blood volume to a level such that the infusion will result in a greater than 1 Gm. per cent drop in hemoglobin concentration;

thus the determined volume will be reasonably accurate. In other words, the method allows one to determine the clinically important point of whether or not the individual is significantly hypovolemic. Error at larger volumes is of less practical clinical significance. It should be emphasized that in cases of severe chronic anemia rapid infusion of 500 ml. of Intradex would of course be dangerous and the method definitely contraindicated.

The estimation of the blood volume from clinical signs and a routine hemogram can be difficult. The described method has been found to be a relatively simple means of gaining some knowledge of a patient's blood volume status. To correct for a blood volume deficit requires knowledge of both the immediate (measured) blood volume and the normal volume for that patient. The latter will usually have to be estimated by some indirect means such as height, weight, or body surface area, and there is a 10 per cent standard deviation in these estimations of normal volume. The accuracy of any blood volume determination cannot affect this inherent 10 per cent error in the application of the results to replacement therapy. From a practical standpoint it would seem that extreme accuracy in blood replacement is unnecessary except in infants. The described method has allowed us sufficient clinical knowledge of a patient's blood volume to outline a rational blood replacement program. The information gained has been used to correct the volume to a safe level. This method allows one to avoid overtransfusion in the elderly and cardiac patient (by choosing the lower range of estimated normal volume) and undertransfusion, where the correction is to only a dangerous preshock level, from which further hemorrhage is likely to result in catastrophic collapse. Our experience with the method leads us to suggest that it is sufficiently accurate for clinical use.

Appendix: Clinical examples

A. External hemorrhage—no hemodilution: Case A-1. A 26-year-old man with recent compound fracture of the left tibia had blood pressure 160/100, pulse rate 78 per minute, and hemoglobin 15.2 Gm. per cent. Blood volume by determination (B.V.D.) was 3,200 ml. Estimated normal blood volume by weight (B.V.W.) was 4,900 ml. Therefore, volume deficit was approximately 4,900 – 3,200 ml., or 1,700 ml. As approximately 700 ml. was lost at the subsequent operation, the total blood loss was at least 2,400 ml. The first postoperative day there was some oozing and hematoma formation. The blood therapy and hemoglobin pattern are shown in Table III.

B. Internal bleeding—no hemodilution: Case B-1. A 47-year-old woman had a pelvic exenteration for stage IV carcinoma of the cervix. During the operation she received 6,000

Table III. Blood therapy and hemoglobin pattern: Case A-1

	Hospital day							
	1	*2*	*3*	*4*	*7*	*9*	*11*	*16*
Hemoglobin concentration	15.2 13.0	12.4	11.8		14.4	14.4	14.0	13.6
Whole blood (ml.)	1,600*	400	400	400				
Intradex	540 ml.†							

*The preserved blood used in this study contained 380 ml. of whole blood and 120 ml. A.C.D. solution per unit.

†In the early part of the study 540 ml. of Intradex was infused.

Table IV. Blood therapy and hemoglobin pattern: Case C-1

	Hospital day									
	1	*2*	*3*	*4*	*5*	*7*	*9*	*12*	*14*	*16*
Hemoglobin	8.0									
concentration	6.7	9.7	10.9	12.8	13.2	10.9	10.9	12.4	12.8	12.4
Whole blood (ml.)	400	1,200	800				400			
Intradex	500 ml.									

ml. of blood. The following morning clinical shock developed. B.V.D. was 1,900 ml. (Hgb_1 was 14.0 and Hgb_2 was 11.05 Gm. per cent). B.V.W. was 3,200 ml. Therefore, blood volume deficit was 1,300 ml. Following 1,200 ml. of blood the blood pressure stabilized and the hemoglobin concentration rose to and remained at 14.4 Gm. per cent.

One week later she had an episode of hypotension; blood pressure was 60/? and pulse rate 160 per minute. The clinical impression was hypovolemia from pelvic bleeding. Following 800 ml. of blood the blood pressure rose to 120/80 but the pulse rate persisted at 140 per minute. B.V.D. after these transfusions was found to be 3,100 ml. As the B.V.W. was 3,200 ml. it was felt that the hypotensive episode and persistent tachycardia were not due to significant hypovolemia. An electrocardiogram showed paroxysmal tachycardia. Following digitalis therapy the pulse rate gradually became normal and there were no subsequent episodes of hypotension. The hemoglobin concentrations were 14.4 Gm. per cent at 1 and 2 days after the last blood volume determination and 14.8 Gm. per cent 5 days later, indicating that she was probably neither hypo- nor hypervolemic.

C. Internal hemorrhage—with hemodilution: Case C-1. A 69-year-old man with a history of coronary insufficiency had massive hematemesis and melena from a duodenal ulcer; blood pressure 100/60, pulse 88 per minute, and hemoglobin 8.0 Gm. per cent. B.V.D. was 2,600 ml., and B.V.W. 4,900 ml. The blood volume deficit was 4,900 – 2,600 ml., or 2,300 ml. The plasma volume increase due to hemodilution was $2{,}600 - (2{,}600 \times \frac{8.0}{14.0})$ ml., or approximately 1,100 ml. (the hemoglobin level on previous admission was 14 Gm. per cent). Therefore, the total amount of blood necessary to return the blood to full volume with a hemoglobin level of 14 Gm. was 2,300 + 1,100 ml., or 3,400 ml. The blood therapy and hemoglobin concentration pattern are shown in Table IV.

On the third hospital day the blood pressure was 110/70 and pulse rate 80 per minute. There was no evidence of further bleeding. As he did not receive all the calculated blood requirement the hemoglobin did not rise to the level of 14 Gm. on which the calculation was based.

Case C-2. A 49-year-old woman showed clinical shock 15 hours after a cholecystectomy. The blood pressure was unobtainable and the pulse thready and weak. She was given 1,000 ml. of blood and an intravenous Levophed drip, but her condition remained poor. B.V.D. at

Table V. Blood therapy and hemoglobin pattern: Case C-3

	Postoperative day						
	1	*2*	*3*	*4*	*5*	*6*	*13*
Hemoglobin	12.1						
concentration	10.6		13.2	15.2		15.2	15.2
Whole blood (ml.)	400	400	400				
Intradex	500 ml.						
Reticulocyte count						1.4	

this time was 2,700 ml., B.V.W. was 3,600 ml. The blood volume deficit was 3,600 – 2,700 ml., or 900 ml. The amount corrected by hemodilution was $2{,}700 - (2{,}700 \times \frac{11.3}{14.4})$ ml., or 600 ml. (admission hemoglobin 14.4 Gm. per cent). The total blood requirement was 900 + 600 ml., or 1,500 ml. Thus the total blood loss since the operation must have been about 2,500 ml. (required 1,500 plus 1,000 ml. already transfused). At operation 2,300 ml. of sanguinous fluid and about 700 ml. of blood clot were found.

Case C-3. A 59-year-old man was admitted because of a bleeding peptic ulcer. He received 4,400 ml. of blood before and during subtotal gastrectomy. B.V.D. one day postoperatively was 3,500 ml. (Hgb_i 12.1 and Hgb_f 10.6 Gm.). B.V.W. was 4,000 ml., so that after Intradex infusion his condition was at or near full volume of blood of hemoglobin 10.6 Gm. per cent. Assuming his normal hemoglobin concentration to be 15 Gm. per cent then the amount of this volume, corrected by hemodilution, was approximately $4{,}000 - (4{,}000 \times \frac{10.6}{15})$ ml., or 1,200 ml., or, conversely, 1,200 ml. of whole blood of hemoglobin 15 Gm. per cent* was required to bring the hemoglobin concentration to 15 Gm. per cent.

Blood therapy and postoperative hemoglobin concentration are shown in Table V.

The authors wish to thank Dr. C. Gardiner and Miss C. Couvier, R.T., Queen Mary's Veterans Hospital, Montreal, for the performance of the radioactive iodinated serum albumin blood volume determinations, and Dr. C. E. Harris and Miss M. Valiquette, R.T., St. Mary's Memorial Hospital, Montreal, for their valuable assistance.

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Laboratory methods

A constant volume fractional sample collector for cardiac output determinations

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Cardiac output is frequently determined by the continuous registration of the changing concentration of dye or of radioactive particles in blood diverted from a peripheral artery. Precordial counting techniques employing a radioactive indicator are also used. Both procedures eliminate the laborious manipulation and handling of the many blood samples obtained with the older multiple fractional sample methods. Nevertheless, the fractional sample technique requires less indicator and usually affords greater accuracy. Precordial counting methods are not suitable for measurements on exercising subjects. A special sample collector was devised to simplify the determination of cardiac output by fractional sample collection. It provides discrete blood samples of a constant volume regardless of variations in arterial pressure. Time-consuming preparation of the many individual blood samples is greatly reduced. Flow measurements in circulation models and comparisons with direct Fick estimates in animals confirmed reliability of the device.

The indicator-dilution method for the determination of cardiac output is in widespread use. Radioactive substances or protein-binding dyes are the indicators most commonly used. Their time-course through the circulation can be measured by 3 methods: (1) diverting a portion of the flow from an artery through a cuvette in which the concentration of the indicator is measured, (2) placing an appropriate detection device externally over the aorta or heart and recording the passage of radioactive material, or (3) collecting multiple arterial blood samples at 1 to 2 second intervals and measuring the concentration of indicator in each sample.[1] With continuous recording methods more indicator

From the Department of Medicine, Northwestern University Medical School, Chicago, Ill.
Supported in part by grants from the American Heart Association, the Illinois Tuberculosis Association, and the United States Public Health Service (HT-5017).
Received for publication Dec. 21, 1960.
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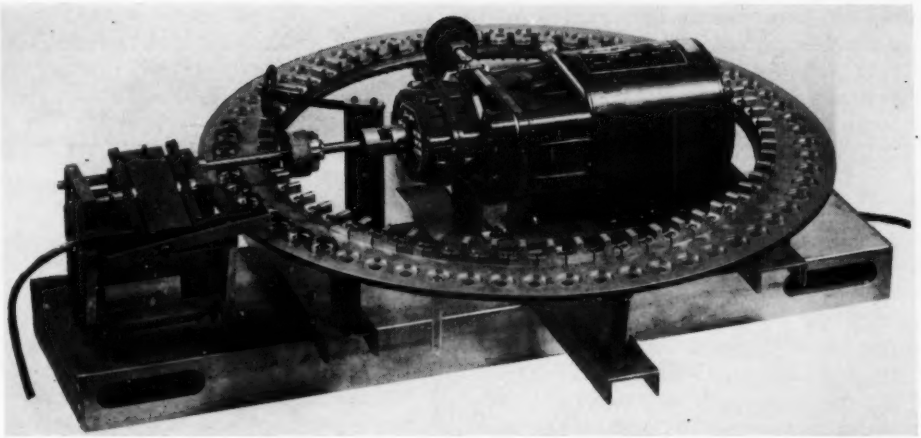

Fig. 1.

is needed than with fractional sample techniques, and repetitive measurements may not be possible because of the rapid accumulation of large quantities of indicator.[2-14] External radioactive counting methods require a constant relationship between the patient and the detection device and thus they cannot be used during exercise. Multiple sample techniques are of proved reliability, and require less indicator than continuous recording methods, but the necessary manipulation and measurement of the numerous blood samples is burdensome.[1, 2]

A new mechanical sample collector* was developed for use in cardiac output measurements. It provides multiple arterial blood samples of a constant volume and, together with radioactive counting equipment, greatly facilitates cardiac output measurements.

Materials

The constant volume fractional sample collector is shown in Fig. 1. It consists of 3 major parts: (1) a set of 3 compression arms, (2) a wheel on which the blood samples are collected, and (3) the motor. There is a variable speed control on the motor with which the sampling rate can be adjusted from 1 sample in 3 seconds to 2 samples per second. With minor modifications samples can be collected at any slower rate. The sample wheel contains a series of adjacent depressions into which cupped planchets, commonly used for radioactive counting, are placed. The center of each depression is drilled out to accommodate a small test tube. A maximum of 60 separate samples—in either planchets or test tubes—can be accommodated.

A short piece of rubber tubing rests in a trough under the compression arms. It connects an indwelling arterial needle to a curved outlet nozzle directly over the planchets or test tubes. An adjustable support bracket attached to the compression unit permits proper positioning of the outlet nozzle. The rubber tubing is occluded by the 3 compression arms in alternate fashion such that the blood contained therein is "milked" into the collecting vessels. The middle arm—2 inches wide—expels the sample, and the other narrower arms serve to prevent the backward reflux or premature ejection of blood. Cams attached to the motor shaft advance the sample wheel in an intermittent fashion. The cams are so positioned that there is a discrete ejection of blood when the sample wheel is stationary; the segment of

*Custom Engineering and Development Co., St. Louis, Mo.

Table I. Continuous flow model comparison

Measured (ml./min.)	*IHSA (ml./min.)*	$\frac{IHSA}{measured} \times 100$ *(%)*
1,190	1,115	94
1,455	1,425	98
1,550	1,515	98
1,730	1,795	104
1,745	1,715	98
1,770	1,710	97
1,900	1,830	96
1,920	1,920	100
1,960	1,970	101
2,295	2,300	100
2,335	2,280	98
2,490	2,520	101
2,530	2,390	95
2,710	2,740	101
2,720	2,630	97
2,760	2,630	95
2,900	2,800	97
3,010	2,970	99
Mean 2,165	2,125	98%
		S. D. ± 7.9%

rubber tubing under the middle compression arm fills with blood while the sample wheel advances, and the next discrete sample is then ejected. At one of the 60 positions, the sample wheel remains stationary despite rotation of the motor shaft. This permits the collection of successive samples in a single container. Collecting 10 to 20 such consecutive samples in a graduated container provides a convenient and reliable method for calculating the volume ejected per sample. There is no residual blood under the compression arms. The volume ejected is constant, regardless of variations in the input pressure within a range of 0 to 300 mm. Hg, provided latex rubber tubing of 3/16 inch inside diameter and 1/16 inch wall thickness is used. The volume ejected per sample is approximately 0.5 ml. of blood. Larger or smaller volumes can be obtained by substitution of a different sized central compression arm.

Radioactive (I^{131}) iodinated human serum albumin (IHSA) was used as the indicator. All samples were collected in cupped planchets previously prepared by the addition of a small quantity of anticoagulant. The radioactivity was determined promptly after collection. Whenever the counting was delayed for a few hours, precautions were taken to prevent evaporation which would alter the geometry of the sample and detector and perhaps change the count rate. An automatic sample changer, a scintillation detector with a 1 inch thallium-activated sodium iodide crystal, a scaler, and a recording timer were available for most of the studies. Each sample was counted to an accuracy of approximately 1 per cent. An occasional flow determination was performed by collecting the samples in test tubes instead of planchets and measuring the radioactivity in a well counter. All injections of indicator were made with a 2 ml. metal-tipped glass syringe held in a metal frame.* Several 1¼ inch 18 gauge thin-wall needles with styli were purchased at one time and carefully examined to insure that they were identical in length and had interchangeable styli. All injections into the circulation models or animals were made through these needles. A second injection with the same syringe in the same metal frame but with a fresh needle was made into a 1 L. volumetric flask, the flask filled to the mark with water, and a suitable volume transferred by a

*Northern Tool Instrument Co., Long Island City, N. Y.

measuring pipette to several planchets or test tubes for counting along with the samples. The metal syringe holder was then disassembled for cleaning. The holder can be adjusted to deliver whatever volume is desired, but adjusting the isotope concentration such that approximately 1.5 ml. is delivered afforded the best compromise between accuracy and rapid delivery.

Methods

Validation of apparatus and results

1. VOLUME PER SAMPLE. To determine if the volume of each sample ejected by the compression unit varied significantly with changes in pressure at the sampling site the following experiment was made: Two microcuries of IHSA was added to a 500 ml. flask of tap water. The bottle was suspended at a height equivalent to approximately 300 mm. Hg hydrostatic pressure from the sample collector. After isotope and water were thoroughly mixed, a stream of the fluid was allowed to run slowly through a rubber tube into a beaker. A needle was thrust through the tubing wall and 10 consecutive samples from the flowing stream were obtained with the sample collector. The procedure was repeated with the water reservoir at the same level as the sample collector. The radioactivity of the samples collected at the 2 levels of the reservoir were compared.

There was no significant difference in the volume ejected per stroke at the 2 pressures used (0 and 300 mm. Hg). The mean net count rate of 10 samples obtained with the reservoir at the high pressure was 496 ± 16 counts per minute (c.p.m.). With the reservoir at zero pressure the net count rate of 14 consecutive samples was 489 ± 21 c.p.m. The difference between the means was statistically insignificant ($P < 0.3$). The measured volumes of 20 additional consecutive samples collected at each pressure were identical. With hydrostatic pressures greater than those used, or with thinner walled rubber tubing in the compression unit, some variation in the volume ejected per stroke was noted.

2. QUANTITATIVE FLOW MEASUREMENTS. A simple model was used to test the validity of this device for quantitative flow measurements. A water reservoir, at constant temperature and pressure, was connected to a mixing chamber filled with glass beads. The outflow passed through a simulated vascular bed of branched rubber tubes which rejoined into a single outlet. Two to 5 μc of IHSA were injected into the circuit between the reservoir and mixing chambers. Samples were obtained at 1 second intervals from one of the branching tubes. The flow was measured directly by collecting the effluent in a graduated cylinder during a carefully timed interval. Flow rates were varied between 1 and 3 L. per minute. This is referred to as the continuous flow model.

In Table I the results of 18 comparisons between measured and calculated flow rates are shown. In these continuous flow model comparisons flow rates from 1.19 to 3.01 L. per minute were achieved. The percentage of the calculated compared with the measured flow is shown in the final column of the table. The sample collector underestimated the flow by an average of 2 per cent. The standard deviation from regression of the IHSA values on the measured flow data was 54 ml. Thus the calculated flow fell within 108 ml. of the measured flow within the range tested 95 per cent of the time.

3. RECIRCULATION FLOW MEASUREMENTS. In order to simulate the normal circulation more closely, a pump replaced the fluid reservoir of the previous experiment, creating a closed circuit (Fig. 2). A second pump was used to divert a portion of the flow from the branching circuit back into the main stream to provide recirculation of the indicator before the primary wave completely passed the sampling site. Direct measurements of the total flow were made by breaking the circuit and measuring the effluent as described above. Special precautions to eliminate all air bubbles from the closed circuit were necessary for reliable measurements. Ten comparisons (Table II) were made at flow rates between 1.84 and 3.31 L. per minute. The sample collector underestimated the flow by an average of 5 per cent. The standard deviation from regression of the IHSA on the measured flows was 186 ml. under these circumstances. A time-concentration graph of the IHSA indicator from the recirculation model is shown in Fig. 3. Its similarity to graphs derived from results with animals and man is apparent.

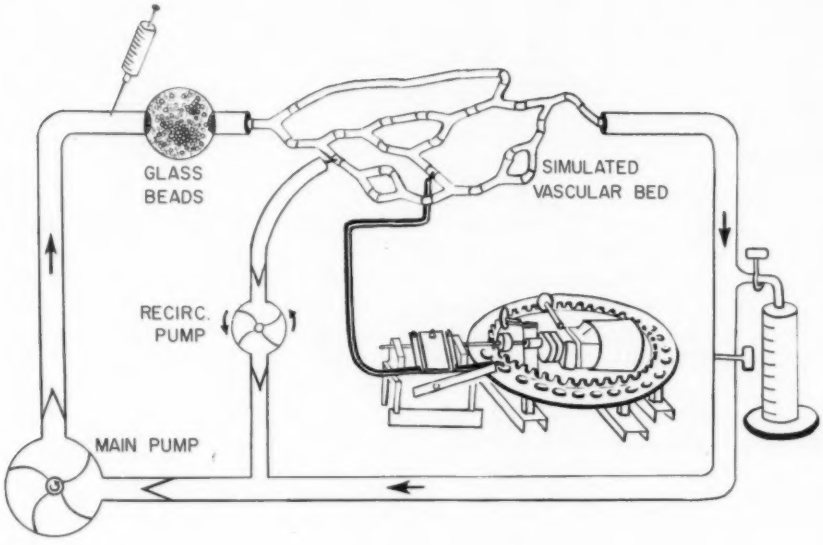

Fig. 2.

4. DIRECT FICK COMPARISONS IN DOGS. An additional validation procedure consisted of simultaneous direct Fick and our indicator-dilution cardiac output determinations in dogs. In anesthetized intubated dogs a cardiac catheter was passed via the jugular vein into the pulmonary artery under fluoroscopic control. Indwelling needles were inserted into both femoral arteries and a forepaw vein. For the direct Fick measurements blood was sampled simultaneously from the pulmonary and femoral arteries during the first minute of the expired air collection. Immediately following the blood collections, the isotope was injected and multiple arterial samples were obtained. Air samples were analyzed for oxygen and carbon dioxide on a Scholander analyzer and blood oxygen determinations were made in duplicate by the Van Slyke apparatus. Usually, 4 consecutive cardiac output measurements were made on the same animal and at least 30 minutes were allowed between determinations. With repeated measurements the cardiac output often declined. This could be obviated by blood or

Table II. Recirculation flow model comparison

Measured (ml./min.)	*IHSA (ml./min.)*	$\frac{IHSA}{measured} \times 100$ *(%)*
1,845	1,745	95
2,140	2,040	95
2,170	2,020	93
2,230	2,340	105
2,270	1,880	83
2,370	2,395	101
2,450	2,395	98
2,760	2,295	83
3,100	3,000	97
3,310	3,170	96
Mean 2,465	2,328	95%
		S. D. ± 6.9%

Table III. Fick versus IHSA: comparison in dogs

Fick (*ml./min.*)	*IHSA* (*ml./min.*)	$\frac{IHSA}{Fick} \times 100$ (%)
520	590	114
530	750	142
770	740	96
870	810	93
910	1,030	113
910	840	92
960	900	94
1,020	970	95
1,050	1,070	102
1,130	1,320	117
1,230	1,390	113
1,400	1,580	113
1,420	1,150	81
1,650	1,860	113
1,760	1,610	92
1,950	2,060	106
1,980	1,920	97
2,020	1,640	81
2,260	2,050	91
Mean 1,281	1,278	102%
		S. D. ± 17.2%

dextran infusions. Some of the animal data were discarded because of respiratory quotients below 0.6 suggesting an air leak or unsteady state, or a prolonged, flat isotope time-concentration curve which precluded accurate output calculations.

Nineteen measurements suitable for comparison are listed in Table III. The range of flows was 0.52 to 2.26 L. per minute. In 9 of the 19 trials the sample collector overestimated the flow; in the remainder it was underestimated. As might be expected the agreement was not as good as noted with the circulation models. Calculation of the regression equation of one method upon the other was not attempted because the error of the Fick method probably approximated and may exceed that of the fractional sample IHSA technique.[2]

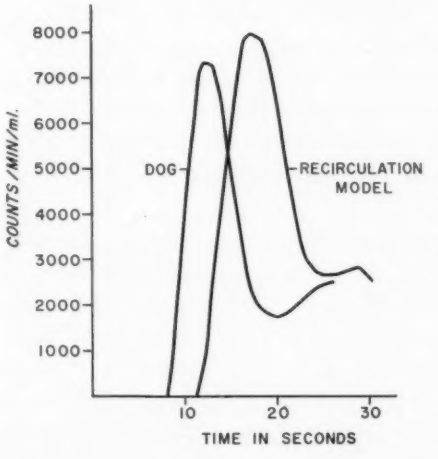

Fig. 3. Time concentration graph of the IHSA indicator from the recirculation model.

Discussion

Radioisotope indicator dilution methods are increasing in popularity,[3-14] particularly with external detector placement.[3-11] Validation has been made on the basis of the following: (1) agreement with simultaneous Fick determinations,[3-8] (2) concomitant arterial sampling methods,[8-11] and (3) results comparable to those previously obtained.[12-14]

Nevertheless, external counting methods are not universally considered reliable. The errors of the method in both theory and technique have been thoroughly investigated by Carter and associates.[15] Because of the problems of collimation and detector placement, and the difficulty in extrapolating the downstroke of the disappearance curve obtained with precordial counting, Carter concluded that the method is unsound. Irrespective of the validity of precordial counting techniques, 2 additional disadvantages of the method merit comment: (1) the dosage of radioactive material needed for each determination is from 5 to 20 times that required if multiple arterial blood samples are removed, and (2) external detection methods cannot be used under any circumstances other than complete rest.

Aspirating arterial blood at a constant flow rate past a light source or a radiation detector circumvents some of the objections created with external measurements, but creates additional technical problems. For accurate counting of radioactive particles in flowing blood a well-shielded, thus a heavy, bulky detector must be located as close as possible to the artery from which the blood is diverted.[16, 17] To detect rapid changes of radioactivity, the volume of blood in proximity with the detector should be small and the rate meter set for a relatively short time constant.[15] With a small portion of the total blood volume being counted at any one time, the total quantity of radioactivity injected is necessarily larger than when counting can be performed slowly. Although some have claimed that 5 μc of IHSA suffice,[12, 14] the majority use 20 to 100 μc, which limits the number of consecutive determinations possible with this technique.[3-5, 7-10, 13]

The numerous disadvantages of continuous recording methods for both dye substances and radioactive material prompted us to return to the original multiple arterial sample collection technique. This seemed to be the most suitable method for accurate, multiple cardiac output determinations in exercising subjects. The constant volume fractional sample apparatus was designed to circumvent the major difficulties of this procedure—preparation and handling of the numerous samples. By ejecting a constant volume into each collecting tube or planchet, one of the most time-consuming features of the multiple sample method—quantitative transfer of an aliquot of plasma, serum, or whole blood—was obviated.

Previously available sample collecting units consist of a series of adjacent containers moving past a continuously flowing blood column diverted from a convenient artery.[18] Sampling is continuous, a new container moving into position every 2 or 3 seconds. The fractional sample collector described here differs in that the arterial stream is sampled for only two-thirds of the time interval between samples. With the sampling frequency at 1 per second, two-thirds of a second is utilized to eject the blood and the balance is used to refill the tubing under the compression arm and to advance the sample wheel to its

next position. Discontinuous sampling might not faithfully detect rapid concentration changes, but this potential error can be minimized with rapid sampling rates. Within the range of flows encountered in dogs and in the circulation models, the 1 per second sampling rate appeared to be adequate for accurate flow determinations. A small systematic difference between the IHSA fractional sample flow determination and the direct measurements in both circulation model experiments is apparent. Why this was so is not clear. A loss of IHSA from the system should result in a greater, not a lesser, value.[2] No systematic difference was noted between the direct Fick and IHSA data.

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A hydraulic pressure generator for testing the dynamic characteristic of catheters and manometers

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A hydraulic pressure generator is described which consists of a loudspeaker system exerting a sinusoidal force on a rubber membrane, the former being driven by a low frequency voltage generator, and a transistor power amplifier. The rubber membrane forms a wall of a small water-filled chamber to which the catheter is connected.

The conditions which must be fulfilled for a reliable operation are discussed.

The fluctuating pressure of the arteries in the heart is often measured by means of a catheter connected to a manometer. Not only must the manometer be able to follow the rapid fluctuations (0 to 8 cycles per second in the human adult), but also, in combination with the catheter, the manometer must guarantee an undistorted transmission.

In order to test manometers or catheter-manometer combinations a hydraulic pressure generator has been constructed. The generator produces sinusoidal pressure fluctuations of variable amplitude and frequency, the amplitude being independent of the frequency. The response of the manometer and catheter is measured as a function of the frequency.

Materials

Constructive details. A schematic drawing of the pressure generator is shown in Fig. 1. A sinus voltage generator (Peekel, 0.15 to 15,000 c.p.s.) with constant amplitude (accuracy within 1 per cent) drives a transistorized powerstage, as shown in Fig. 2.

The powerstage is connected at the output to a loudspeaker system (7 Ohm) having a weak metal diaphragm attached to the solenoid. The solenoid is able to exert a force against a metal plate *B*, glued to a weak rubber membrane *C* at the base of the vessel *D*, which is filled with water. The total compliance of the serially connected membranes is about 100 mm.^{3} per 10 cm. Hg., which is low enough for our purpose (see next paragraph). The constant

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Received for publication July 18, 1960.

voltage amplitude of the sinus generator gives rise to a current with constant amplitude in the solenoid. This current causes a force on the plate *B* proportional to the current. Thus a pressure in the water in *D* is evoked which is proportional to this force and, hence, proportional to the solenoid current. The linearity is maintained up to 1 amp, the maximum permissible solenoid current. The conversion factor is 92 mm. Hg per ampere. The maximum displacement of the solenoid is about 0.5 cm. The area of the vessel base is 2 cm.^{2}, hence a volume of 0.5 cm.^{3} can be displaced without deviation from linearity.

The catheter can be attached to the top of the vessel *D*. Also, a device consisting of a tube *E*, a balloon *F*, and a sphygmomanometer can be connected with *D*. This device is used for both air-free filling and calibration. As *E* induces a great compliance of the system the generator can be disconnected from *E* at point *G* by means of artery forceps on the plastic connection tube. Of course, a leak-free cock which, however, is not so easily constructed can also be used for this purpose. A static pressure can be made most simply by a d.c. current in the solenoid adjustable by the potentiometer *p* (Fig. 2). The membrane remains then in its medium position. This can be used for fixing a certain mean pressure level. For static measurements it is also possible to use a d.c. voltage source at the input of the powerstage.

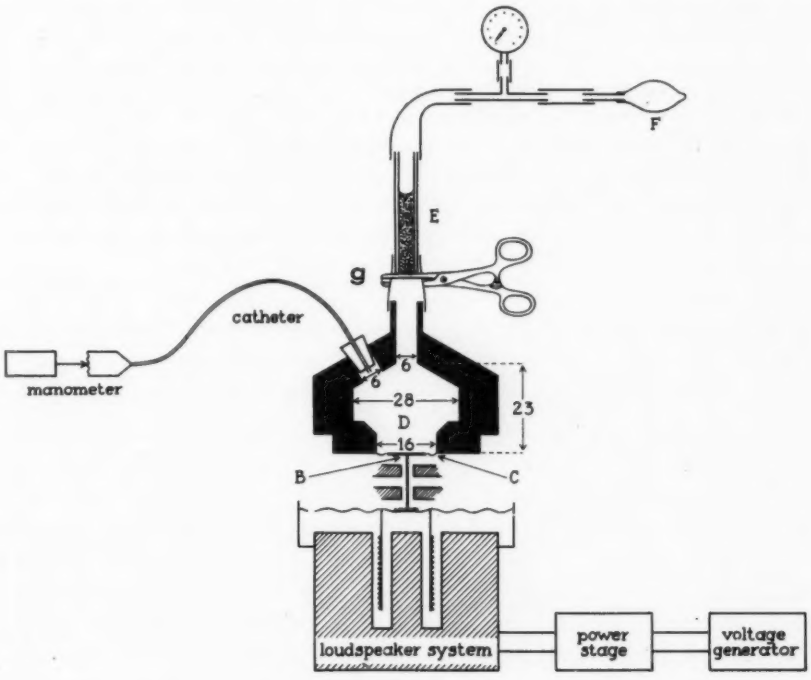

Fig. 1.

The output of the hydraulic sinus generator measured with a manometer with a compliance of 1 mm.^{3} per 10 cm. Hg directly connected to it is undistorted from 0 to 50 c.p.s., which is more than is required. Fig. 3 shows the response characteristic of catheter No. 4 with 1 M. length, connected to a manometer with a membrane compliance of 0.013 mm.^{3} per 10 cm. Hg.

GENERAL REQUIREMENTS FOR A PRESSURE GENERATOR. The main requirement is that the generator must provide a well known sinusoidally varying pressure at the input of the catheter with an amplitude which is independent of the frequency and of the properties of the catheter-manometer system. This will be the case if the generator has an internal impedance which, for all practically important frequencies, is small in comparison with the impedance of the system to be tested.

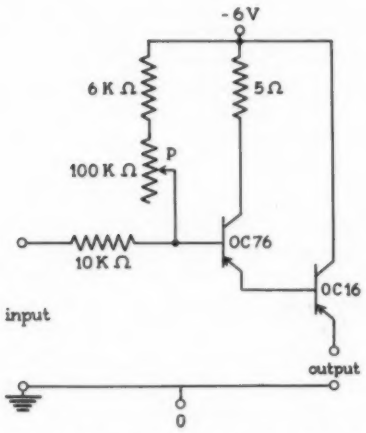

Fig. 2. The transistorized powerstage.

Fully analogous to the electrical impedance, the hydrodynamic impedance consists of a part due to the inertia of the fluid and the moving membranes of the generator, another part due to the stiffness of the membranes, and finally, a part caused by friction. In other words, the sum of the inertial force of the moving fluid in the vessel and membranes of the generator, the restoring forces of the membranes, and the force caused by the viscous resistance of the generator vessel must be small in comparison with the force exerted by the solenoid. In that case the water pressure at the input of the catheter will be practically equal to the water pressure at the base of the vessel of the generator and equal to the solenoid force per unit of membrane area.

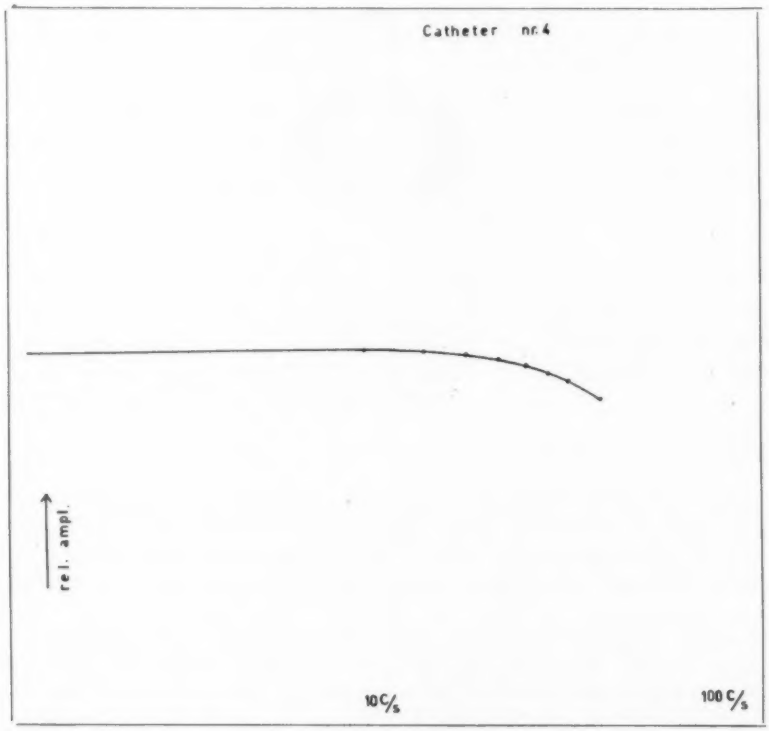

Fig. 3.

The friction forces in the generator are negligible. It is possible also to make a generator in which the restoring forces of the membrane and the inertial forces are sufficiently small.

The restoring force of the membrane may be neglected if the volume displacement is very small. This depends on the input compliance (the inverse of stiffness) of the tested device. The usual types of blood pressure manometers have compliances between 0.01 and 1 mm.^{3} per 10 cm. Hg. Most catheters have lengths of about 1 M. and compliances of 1 mm.^{3} per 10 cm. Hg, irrespective of their diameter. A catheter manometer combination has at most a compliance of 2 mm.^{3} per 10 cm. Hg. Because our generator has a compliance which is 50 times as great (see previous paragraph), the restoring force of the membrane is only 2 per cent of the total force in the worst case and hence can be neglected.

The inertia forces begin to play a role at the high frequencies if the hydraulic impedance of the tested system at the input is low, i.e., if the streaming velocity of the water is great. This velocity can be kept small if the bore of the tube of the vessel is great. The dimensions of the pressure generator are such that these inertia forces are much smaller than 1 per cent of the force exerted by the pressure at high frequencies even when a big catheter (No. 8) with a weak manometer (1 mm.^{3} per 10 cm. Hg) is connected.

The impedance of catheters connected to manometers decreases with increasing diameter of the catheter, compliance of both, and frequency. If the impedance of the generator is not small in comparison with the impedance of the system to be tested, the pressure at the input of the system is not known and must be measured separately with a calibrated very stiff manometer.

Another requirement is that linearity must be maintained for large volume displacements of the membrane of the vessel, because the test must be performed in a large range of mean pressures and the pressure amplitude of the generator must be independent of this mean pressure. Our generator is linear up to displacements of 500 mm.^{3}

Conclusions

In the paper by Noble[1] also describing a pressure generator, the question of the internal impedance of the generator is not mentioned. He constructed a pressure generator with the immovable solenoid attached to the core. The gap between an iron plate and the core is 0.25 mm. A d.c. current through the solenoid secures linearity of attraction force of the iron plate. If this linearity will be of the order of 2 per cent then the suction pressure exerted by the d.c. current is 25 times greater than the maximum permissible pressure amplitude of the sinusoidal fluctuations.

If a positive pressure mean level must be maintained, the deflection of the membrane must be more than 25 times its sinusoidal amplitude, which corresponds with a pressure of 50 mm. Hg, peak-to-peak. As the total deflection of the membrane cannot be more than 0.25 mm. it must be rather stiff. This is much more unfavorable than our construction, in which a positive mean level of the pressure can be balanced by a d.c. current pushing against the plate, while in the device of Noble only attraction can occur.

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Rapid detection of specific phage types of staphylococci

ARTHUR WHITE, M.D.* *Louisville, Ky.*

A method of detecting carriers of type 81 staphylococci by dilution of the culture in phage type 81 lysate was useful in rapidly and accurately detecting 17 nasal carriers of type 80/81 staphylococci among 100 patients. In addition, 4 lesions due to type 80/81 staphylococci were identified and a single lesion due to type 3c/71/55 staphylococci could be excluded by the same method.

The various techniques and their relative usefulness for phage typing of staphylococci have recently been reviewed by Blair and Carr.[1] The major disadvantages of this epidemiologic method are the requirements of time, personnel, and equipment and the need to isolate and subculture staphylococci and to standardize the phage inoculum. Because of these requirements, there is often a considerable delay before the results are available in an epidemic.

Initial studies with staphylococcal strains used in propagating bacteriophages have shown that the susceptibility to individual phages could be determined within 24 hours either by initially culturing the strains on the surface of agar media into which phage was incorporated or by diluting the cultures in phage lysates before culturing onto solid media. To evaluate this method further, 100 nasal cultures of hospitalized patients and personnel and cultures of a number of lesions were made in an attempt to identify rapidly those staphylococci lysed by phage 81. This particular phage was chosen because over half of the lesions acquired in this hospital are caused by 80/81 staphylococci.[2]

Materials and methods

Bacteriophages were propagated in heart infusion broth (Difco) with 0.004 M per liter of added calcium.[3] The phage 81 lysates produced confluent lysis of type 81 propagating strain at 1:1,000,000 dilution. Nasal cultures and cultures of lesions were taken

From the University of Louisville Department of Medicine, Louisville, Ky.
Supported by Grant No. E-2561 from the National Institute of Allergy and Infectious Diseases, Public Health Service, Bethesda, Md.
Received for publication Dec. 2, 1960.
*Markle Scholar in Medical Science.

with a cotton swab moistened with heart infusion broth with added calcium; the swab was placed in a culture tube with 3 ml. of heart infusion broth and shaken for 5 minutes on a Kahn shaker. The broth was then diluted in heart infusion broth with added calcium and in lysates of phage 81; 0.1 ml. of each dilution was placed on the surface of heart infusion agar media. After overnight incubation at 37° C., pigmented colonies were enumerated on both sets of plates and a single pigmented colony from each culture was tested for coagulase production and typed with 22 bacteriophages. When staphylococci were present only in cultures diluted with heart infusion broth, the strain was assumed to be a phage type 81 staphylococcus.

The following bacteriophages* were used in this study:

Group I: 29, 52, 52a, 79, 80
Group II: 3a, 3b, 3c, 71, 55
Group III: 6, 7, 42e, 47, 53, 54, 73, 75, 77, Va_4
Unclassified: 81, 42d

Table I. Colony counts of nasal cultures from carriers of staphylococci other than phage type 80/81

Phage type	*Diluent*	
	Broth	*Phage 81 lysate*
71	2.9×10^6	2.3×10^6
52a/79	2.0×10^6	2.9×10^6
Not typeable	2.0×10^6	2.3×10^6
Va_4	2.3×10^5	3.4×10^5
3c/55/71	2.2×10^4	2.2×10^4
47/77	2.0×10^4	1.2×10^4
Not typeable	2.0×10^4	2.1×10^4
52a/79	1.8×10^4	2.3×10^4
6/7/47/54	1.6×10^4	1.6×10^4
$6/47/54/Va_4$	1.3×10^3	1.4×10^3
Va_4	4.2×10^2	3.0×10^3
52a/79/80	1.2×10^2	1.8×10^2
79	6.0×10^1	6.0×10^1

Results

No coagulase-positive staphylococci were isolated from 70 of the 100 patients from whom nasal cultures were obtained regardless of whether or not the cultures were diluted with heart infusion broth or in broth with phage 81. Staphylococci lysed by phages 80/81 were isolated from 17 patients when the samples were diluted in heart infusion broth, but no staphylococci were isolated from samples diluted in phage 81 lysate. The numbers of phage type 80/81 staphylococci isolated varied from 600 to 4,320,000 colonies per culture, with a median value of 28,000.

In the remaining 13 patients a number of different types of staphylococci were isolated in almost equal numbers by both methods (Table I).

Five skin lesions were cultured by the same methods, and, again, no phage type 80/81 staphylococci were isolated from cultures diluted in phage 81 lysate,

*The bacteriophages and propagating strains were kindly furnished by Dr. John Blair, Hospital for Joint Diseases, New York, N. Y.

but 4 of the 5 cultures contained large numbers of type 80/81 staphylococci when diluted in heart infusion broth. The remaining culture contained identical numbers of staphylococci lysed by phages 3b/3c/71/55 when diluted in either heart infusion broth or phage 81 lysate.

Discussion

By dilution of primary cultures in 81 phage lysates, it was possible to detect rapidly and accurately carriers and lesions due to type 80/81 staphylococci. By the criterion stated by Williams and Rippon,[4] staphylococci are assumed to be different if the phage patterns differ by two or more phages. All staphylococci susceptible to phage 81 in this study were phage type 80/81 staphylococci; however, staphylococci susceptible to phage 81 and several other bacteriophages would also be lysed when diluted in phage 81 broth and, therefore, initially would be thought erroneously to be type 80/81 staphylococci. Staphylococci from carriers or lesions which were not susceptible to phage 81 could be readily excluded.

The major advantage of using phage lysate in initial cultures is the rapidity with which most sources of a given phage type staphylococcus can be excluded. With the usual method of phage typing a minimal period of 72 hours is necessary before staphylococci can be isolated, subcultured, and subjected to phage typing, even if all phages have been standardized recently. With phage 81 lysates, most sources can be excluded within 24 hours. The major limitation is the restriction of the method to a single phage; the use of several phage lysates would be too demanding of time and materials.

This method also makes it possible to identify carriers of more than one strain although the second strain might be present in small numbers. In the group of nasal carriers of type 80/81 staphylococci, all staphylococci were susceptible to phage 81 even when as many as 4,000,000 colonies were present. This confirms and extends the observations of Gould and McKillop[5] that usually only one staphylococcus can be isolated from the nose of a given carrier.

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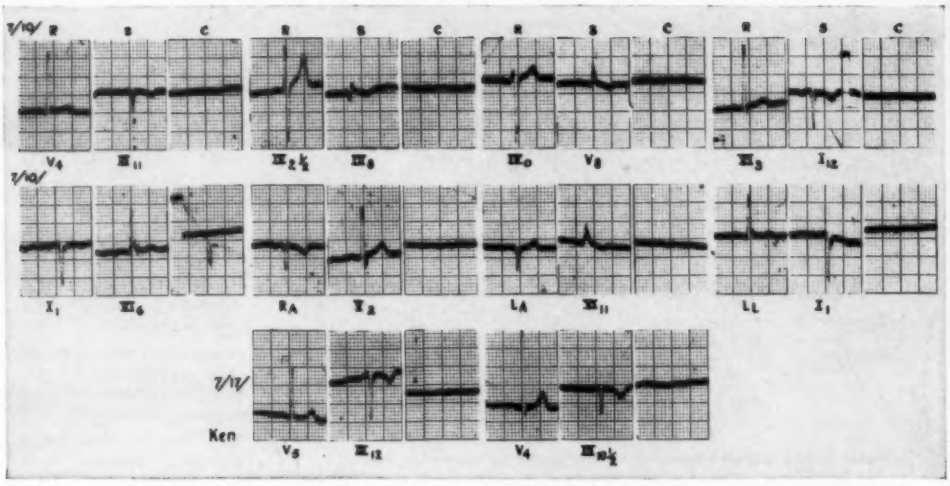

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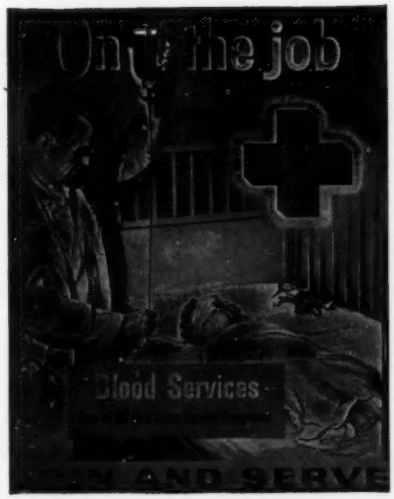

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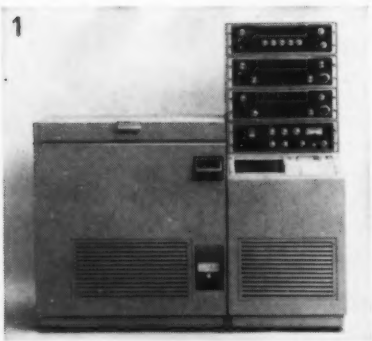

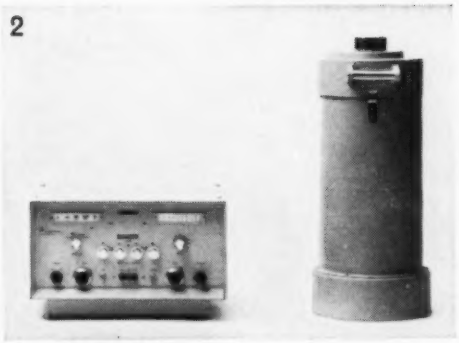

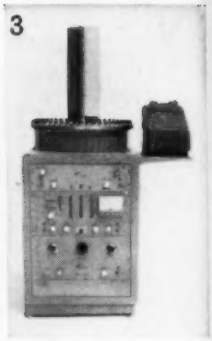

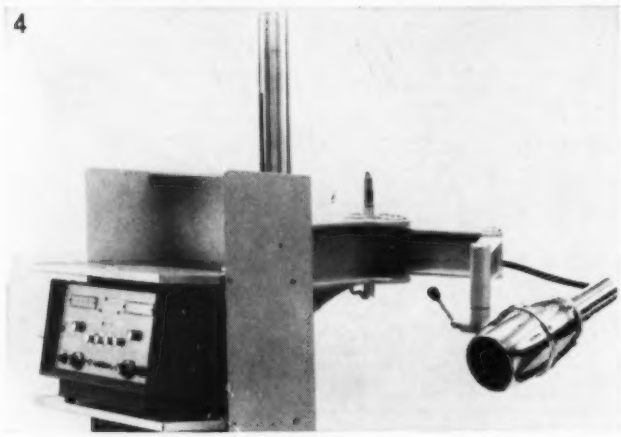

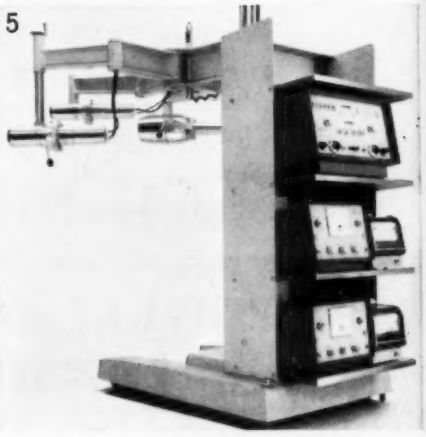

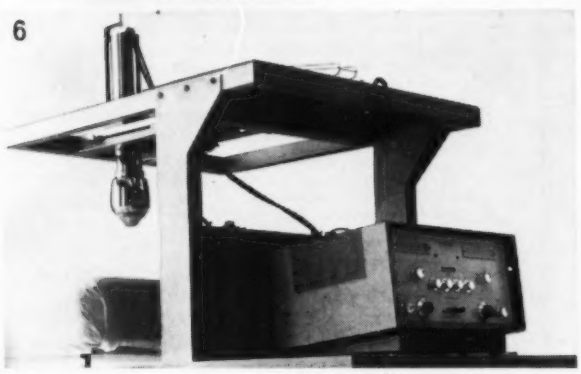

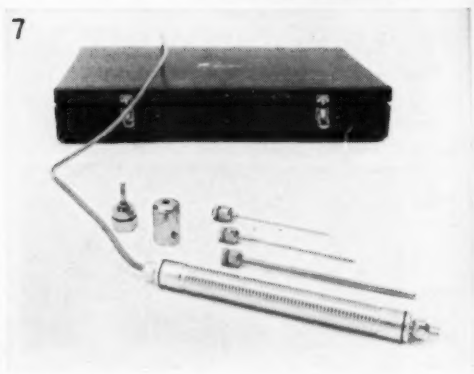

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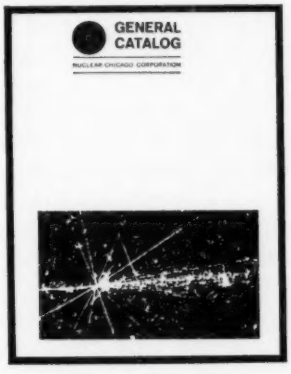

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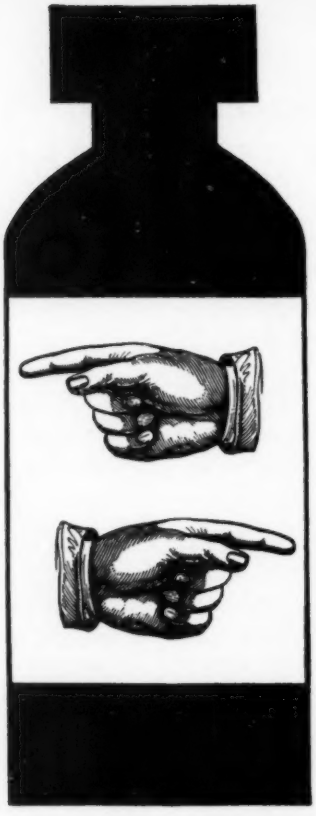

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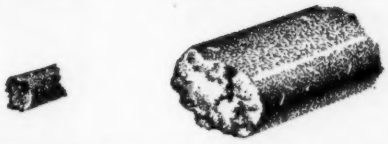

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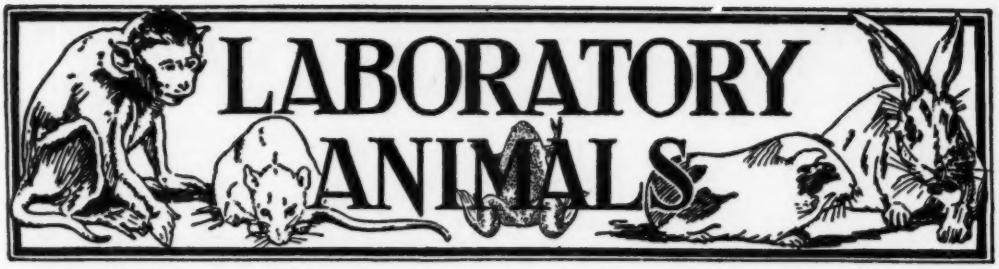

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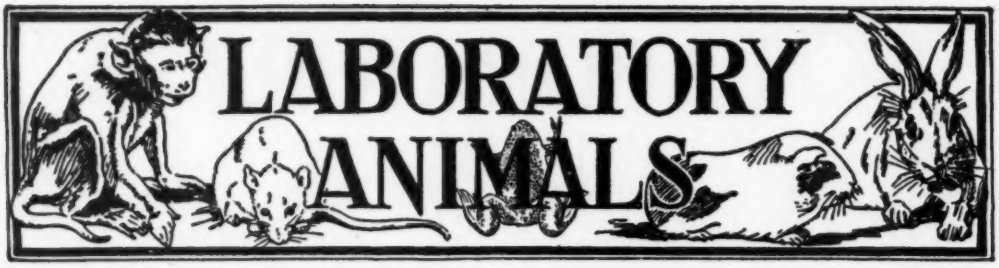

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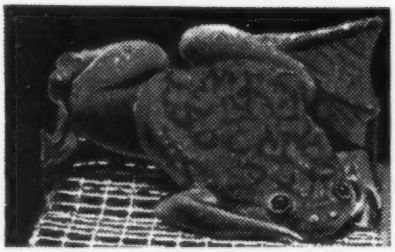

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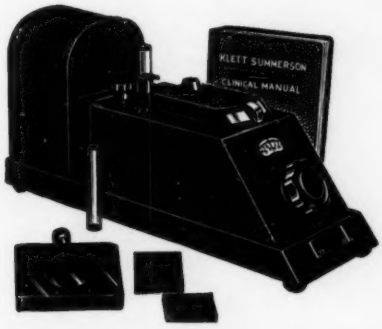

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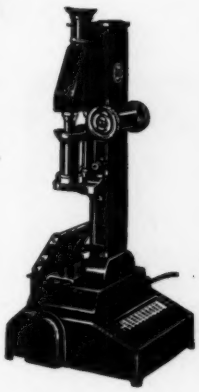

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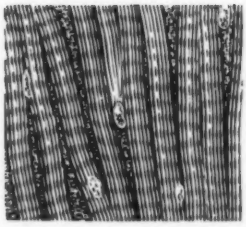
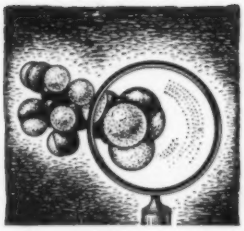


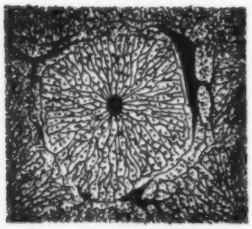
IN AUGUST – (1798) – *Philosophical Magazine* speculates upon the nature of muscular contraction. "It appears very certain that the cause of muscular movement is owing to the nerves . . . But how does the nerve move? Some philosophers have compared its movement to the oscillation of an extended cord which is struck: But. 1. The nerves are not extended: 2. They are enveloped on all sides; whereas the cord has no points of contact but at its two extremities. It appears more probable to others to consider the nerve as . . . a series of vehicles in which flows a fluid called the nervous fluid. I have supposed that this nervous fluid is of a nature analagous to the *aura feminalis*. Others have fought for the cause of irritability in electricity, and the experiments in Galvanism seem to give some weight to this opinion."[1]

Now, a century and a half later, there are still many unanswered questions as to the nature of muscular contraction. There is general agreement, however, on the importance of ATP as the source of chemical energy for muscular movement. Schwarz has long played a leading role in providing ATP and other biochemical phosphates involved in energy transfer reactions – ADP, adenylic acid, adenosine-3′:5′-cyclic phosphate, GTP, GDP, CTP, and CDP. Some of these compounds are available in radiochemical form. In addition, we supply dehydrated firefly tails for precise and specific micro assay of ATP.



IN AUGUST – (1938) – a report from Paris provides insight into the role of sulfhydryl groups in enzymatic activity. It is suggested that changes in the activity of the enzymes may be attributed to oxidation and reduction of SH groups. Rapkine[2] has shown that oxidized glutathione inactivates the enzyme catalyzing the oxidation-reduction reaction between triosephosphate and pyruvate. If the excess glutathione is dialysed away, the enzyme can be reactivated by reduced glutathione or cysteine, the latter being more rapid in its action.

Today, enzyme chemists use sulfhydryl compounds routinely to activate a large number of enzymes. Toxicologists are interested in them as protective agents against radiation, nitrogen mustard, and other metabolic poisons. Glutathione was the first compound made and sold by Schwarz over twenty years ago. We have been interested in sulfhydryl compounds ever since, and now offer oxidized and reduced glutathione, cysteine, cystine, homocysteine thiolactone, thiolated gelatin. Some of these compounds have been labeled with S^{35} or C^{14}.



IN AUGUST – (1953) – a group of Scottish investigators[3] discusses the incorporation of N^{15}-glycine, C^{14}-formate, and S^{35}-methionine into the nucleic acids and proteins of rat liver cell nuclei and cytoplasmic fractions. Nuclear DNA incorporated C^{14} and N^{15} at a slow rate, but nuclear RNA showed greater activity than the RNA of mitochondria, microsomes, or cell sap. With all three isotopes there was no great difference between rate of incorporation into nuclear and into cytoplasmic protein. This indicates that nuclear RNA and protein play an active role in metabolic processes in non-dividing cells.

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1. Irritability of the animal fibre. Philosophical Magazine *1*:307 (Aug.) 1798. 2. Rapkine, L.: Sulphydryl groups and enzymic oxido-reduction. Biochem. J. *32*:1729, 1938. 3. Smellie, R. S.; McIndoe, W. M., and Davidson, J. N.: The incorporation of N^{15}, S^{35}, and C^{14} into nucleic acids and proteins of rat liver. Biochem. et Biophys. Acta *11*:559 (August) 1953.

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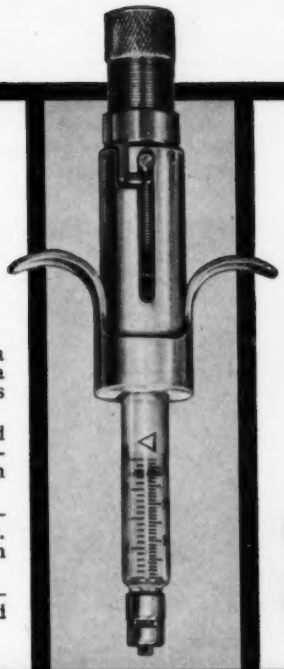

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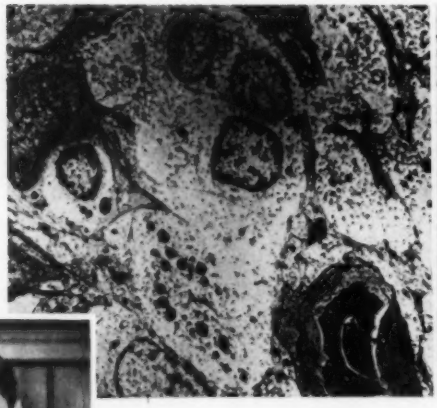
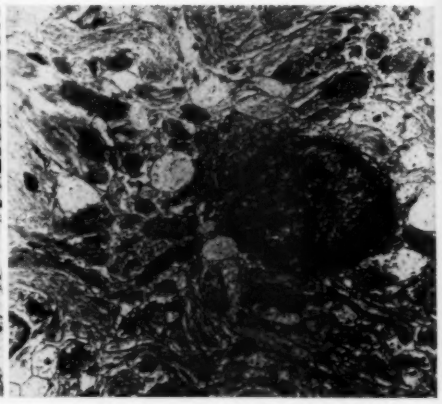
Fig. 1322. Electron micrograph of oligodendroglioma. Several neoplastic cells with abundant pale cytoplasm are arranged near a blood vessel (lower right). Mitochondria are especially prominent in cell at left of blood vessel. (x5,000.) (Courtesy Dr. Sarah Luse.) This illustration appears 40% larger in the book.

Fig. 1325. Electron micrograph. Cellular processes and cells forming this optic nerve glioma are strikingly similar to those of gliosis. From child 6 years of age. (x7,000.) (Courtesy Dr. Sarah Luse.) This illustration appears 40% larger in the book.





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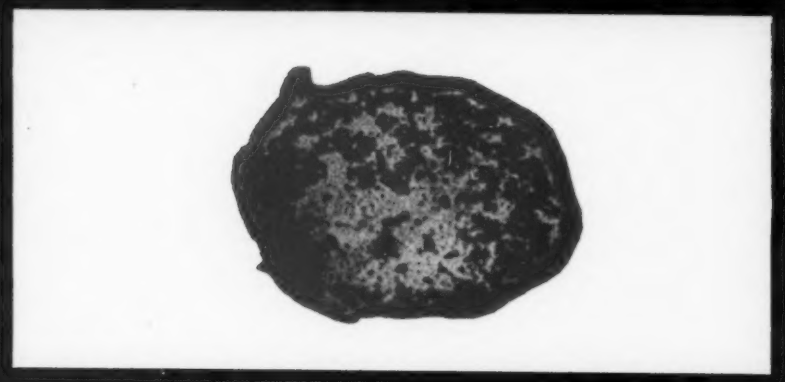

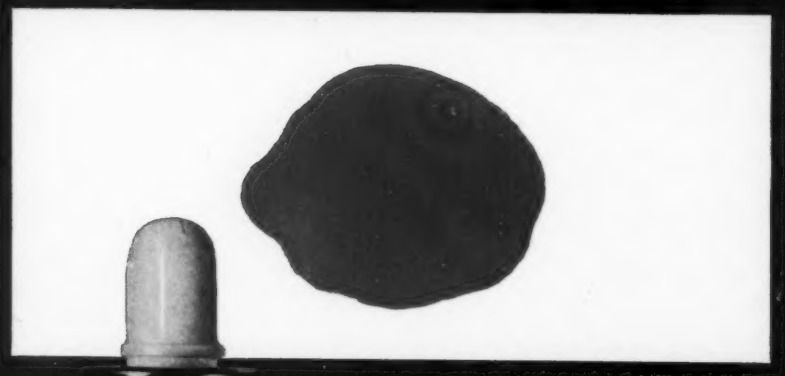

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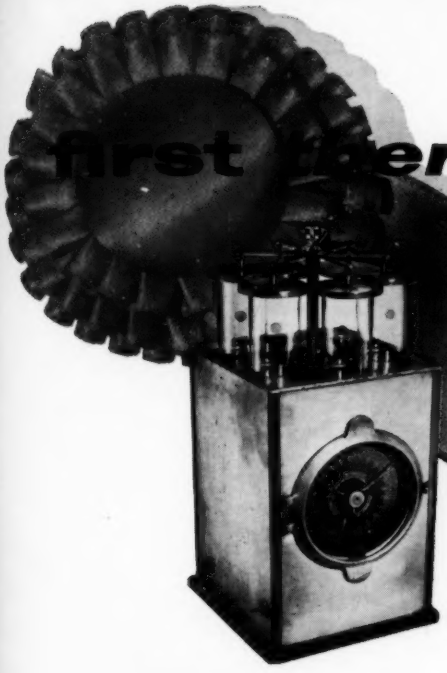

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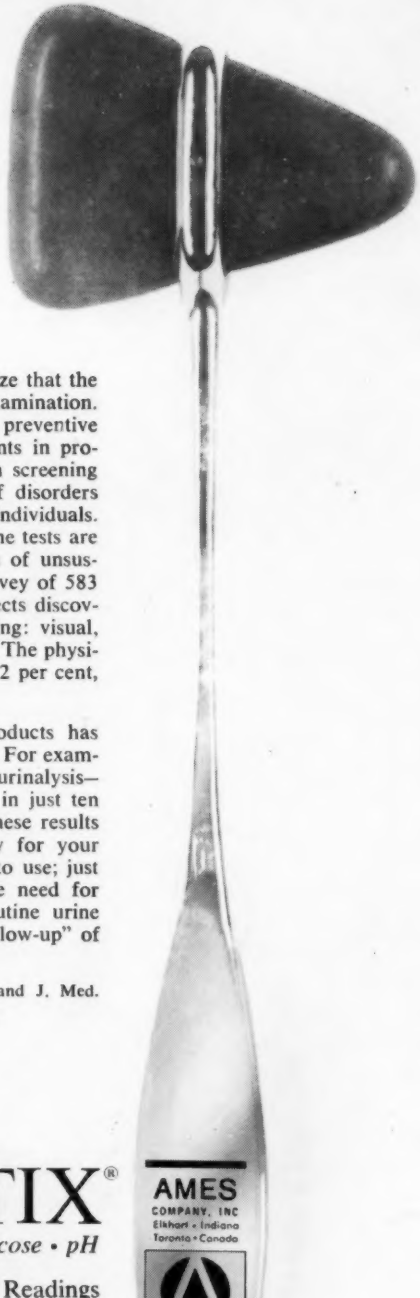

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